PAX 5 EXPRESSION IN LYMPHOMAS AND ITS CORRELATION WITH CLINICAL FEATURES AND OTHER PANEL OF MARKERS IN LYMPHOMAS

DISSERTATION

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CERTIFICATE

This is to certify that the dissertation work entitled "**PAX 5 EXPRESSION IN LYMPHOMAS AND ITS CORRELATION WITH CLINICAL FEATURES AND OTHER PANEL OF MARKERS IN LYMPHOMAS"** submitted by Dr. R.M.LAKSHMI KANTH is the work done by him during the period of study in the department of Pathology, PSG IMS & R from June 2010 to February 2013. This work was done under the guidance of **Dr. S.Shanthakumari**, Professor, Department of Pathology

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CONTENTS

INTRODUCTION

"Lymphomas are malignant neoplasms of lymphoreticular system" as quoted by John K C Chan in the book 'Diagnostic histopathology of tumors' [1]. They present as a solid mass generally. The incidence of this tumor is increasing worldwide as well as in India and is not a rare tumor anymore. The national cancer registry programme in its first all India report in the year 2001-2002 claims an incidence of 5.1 per one lakh population. [2]

Painless lymph node enlargement confined to one lymph node region or involving multiple lymph node regions is the commonest clinical presentation of a lymphoma. Apart from the nodes, lymphomas can involve skin, gastrointestinal tract, salivary glands, nasopharynx, paranasal sinuses, lung, thyroid, ocular adnexa, kidneys, adrenals, breast etc. When the lymphomas involve the sites other than the nodes they are called extranodal lymphomas. [1]

Lymphomas when suspected clinically needs to be confirmed histologically. A confirmatory diagnosis of lymphoma is made by a histological examination, sometimes aided by ancillary techniques like immunohistochemistry, flow cytometry and genetic studies. The lymphomas can be broadly categorised as Hodgkin's lymphoma (HL) and Non Hodgkin's lymphoma (NHL). Based upon the principles adopted in REAL classification, WHO has classified about 58 lymphomas under the following six broad groups. [3]

- Precursor lymphoid neoplasm
- Mature B cell neoplasms.
- Mature T cell and natural killer (NK) cell neoplasms
- Hodgkin lymphoma
- Histiocytic and dendritic cell neoplasm
- Post transplant lymphoproliferative disorders

With the existence of this vast range of lymphomas it is quite essential to diagnose the specific type of lymphoma as they all have different clinical outcomes and different modes of treatment. Diagnosing the type of lymphoma and subcategorizing them is challenging as most of them have an overlapping morphology making it practically difficult to diagnose the type based upon the morphology alone. Hence the aid of ancillary techniques like flow cytometry and IHC becomes essential. IHC is a reliable and most commonly used technique in these situations. IHC has many roles in a case of lymphoma, but the most important role of IHC is in establishing the cell lineage. PAX5 is one such IHC marker helpful in establishing the cell lineage and thereby aid in diagnosing the specific subtype of lymphoma.

PAX5 gene is a member of the paired box gene family and is located in chromosome 9p13. It encodes the transcription factor, PAX5, also known as B cell specific activator protein (BSAP). It is expressed in B lymphocytes from

pro B cells to mature B cells and is essential for B cell development and differentiation and is reliably detected by immunohistochemistry.[4] PAX5 expression is specific for B cells especially in the precursor stage where CD20 is negative. Thereby detection of PAX5 expression in lymphomas is a useful tool for diagnosis and in sub classification of lymphomas. [5][6]

Therefore, we propose to do a retrospective study to assess the PAX5 immunoreactivity as a B cell lineage marker in the samples received as lymphoid malignancies during the period of 2009 to 2010 and to correlate with the other routinely used CD markers in cases of lymphoma.

AIMS AND OBJECTIVES

- To assess the expression of PAX5 in lymphomas and to correlate it with the other pan B cell markers.
- To correlate it with various clinical parameters and other panel of markers for lymphoma

REVIEW OF LITERATURE

Lymphomas are the malignant neoplasms arising from the cells of immune system or the lympho-reticular system.[1] They present as solid masses, unlike leukemia, which also have the similar origin but involves the bone marrow and/or peripheral blood. A global study on incidence and survival rates of various cancers across different global areas done by Parkin DM et al estimated 2,80,000 new cases of lymphomas contributing 3.4% of the total cancer incidence. Lymphomas are responsible for about 2.9% of all cancer related deaths globally. The incidence of lymphomas was very less in the south central Asia when compared to the rest of the world and the maximum number of cases was reported from western countries, Africa and Australia. [7]

Although the incidence of lymphoma is low in India, the five year survival rate is markedly less when compared to the developed countries. The incidence of lymphomas is increasing throughout the world as well as in India. As per the national cancer registry programme: first all India report 2001-2002 the incidence of lymphoma was 5.1 per 1,00,000.[2] Balakrishna B Yeole et al studied the incidence of Non Hodgkin Lymphoma (NHL) in all the major registries in India and observed a significant increase in the incidence of NHL over the years in all the registries.[8] This increasing incidence rates and poor survival makes lymphoma a major concern. In order to facilitate the study of epidemiological and clinic-pathological aspects of lymphomas in India , a

Lymphoma registry has been established in Tata Memorial Hospital, Mumbai.[9] The increasing incidence rate is in part contributed by improving diagnostic methods, evolving criteria for lymphomas, increased incidence in AIDS and AIDS associated lymphomas. Balakrishna B Yeole suggests an extensive study to ascertain the influence of the above mentioned factors on the increasing incidence rates. [8]

Lymphomas show a definite male predominance throughout the world.[7] The age group commonly involved vary with the type of lymphoma. Hodgkin Lymphoma (HL) shows a peak incidence between 11-30 years and between 51-60 years. NHL consists of numerous histologic subtypes and the age groups involved understandingly vary. In general, NHL are more common after 50 years of age.[1] A study of HL and NHL in rural India done by Sudipta Chakraborti et al also found a similar age distribution for HL, but NHL seemed to involve younger patients when compared to the rest of the world.[10]

A non tender enlarged lymph node, involving a single or multiple groups is the most common presentation of lymphoma. They can virtually involve any site, and commonly involved sites other than lymph node are skin, gastro intestinal tract (GIT), salivary glands, spleen, tonsils, sinuses, brain, lung etc. Lymphomas involving sites other than lymph nodes are called extra-nodal lymphomas.[11][1] They can secondarily involve the bone marrow or spill into the peripheral blood and present as leukemia.[11] In addition to the

lymphadenopathy they can have other symptoms like fever, weight loss, anorexia, dyspnoea, abdominal/ chest pain, compressive symptoms, pruritus or even bleeding. There could be an enlarged liver or spleen and anemia associated with lymphomas.[10] They are prone to infections due to loss of immunity. The patients also have predilection to develop auto immune diseases owing to immune modulation and the vice versa is also true. [11]

EVOLUTION OF CLASSIFICATION OF LYMPHOMAS:

The first case of lymphoma was identified by Thomas Hodgkin while working at the Guys hospital, London in the year 1832. He published his paper on lymphatic disease "On some morbid appearances of absorbent glands and spleen". His observations consented with those of Samuel Wilks, who named this condition as Hodgkin's disease, paying credit to Thomas Hodgkin. It was in 1898 and 1902, when Carl Sternberg and Dorothy Reed gave the most important diagnostic entity for Hodgkin's disease by defining the classical Reed-Sternberg cell (RS cell). Variants of RS cells were later identified. [12]

HD was believed to be a benign process progressing into malignancy. The first classification of HD introduced in the year 1944 by Jackson and Parker was based upon this concept. Later in 1966, Lukes and Butler introduced a classification of HD which was shortly revised at Rye conference, where it was accepted to classify HD into 4 subtypes. This classification had a good

correlation with the survival and outcome, making it popular among the clinicians.[12]

The application of immunohistochemical methods for studying various diseases came into light in 1970s. The advent of the immunologic methods resulted in better understanding of the disease process, recognition of new neoplasms and eventually influenced the classification of many tumors. The RS cells and its variants showed positivity for CD15 and CD 30 in most of the cases, but there were a considerable number of cases which were negative for these markers. These cases showed similarity in clinical and morphological features. The Revised European American classification of lymphomas (REAL) introduced in the year 1994, took into consideration the immunological and genetic features in addition to morphology.[13] They categorized HL into classical HL (CHL), which are positive for CD15, CD30 and nodular lymphocyte predominant HL (NLPHL), which are negative for the above markers. The CHL was again categorized into 4 subtypes. The present WHO classification of lymphoma adapts the REAL classification for HL, with a slight modification of including unclassified category under CHL. The following table shows the various classifications used for HL over the years. [12]\

TABLE 1: VARIOUS TYPES OF CLASSIFICATION ADOPTED FROM 1944 TO 2008

YEAR	NAME OF THE CLASSIFICATION	CLASSIFICATION
1944	Jackson – Parker	Paragranuloma Granuloma Sarcoma
1966	Rye conference	Lymphocyte predominant Nodular sclerosis Mixed cellularity Lymphocyte depleted
1994	REAL	Classical HL Nodular sclerosis Lymphocyte rich Mixed cellularity Lymphocyte depleted Nodular lymphocyte predominant HL(NLPHL)
2008	WHO	Classical HL Nodular sclerosis Lymphocyte rich Mixed cellularity Lymphocyte depleted unclassifiable Nodular lymphocyte predominant HL(NLPHL)

The evolution of the classification of the NHL is a little more complex than that of the HL. Ever since the identification of HD, numerous neoplasms arising from the lymphocytes were identified, which were different from the HL in terms of morphology and clinical picture. These tumors were categorized as Non Hodgkin Lymphoma (NHL), simply implying that these tumors are lymphomas other than Hodgkin's lymphoma.

At first, attempts were made to classify NHL based upon the differentiation. Differentiation refers to the degree of resemblance to the normal counterpart. Hence in 1966, Henry Rappaport classified the NHL into lymphocytic, histiocytic, mixed type and undifferentiated types. The lymphocytic type was again classified into poorly differentiated and well differentiated subtypes. With improved understanding of lymphomas, the term histiocytic used in this classification was found to be erroneous as they do not represent the tumors arising from histiocytes. Hence an improved classification became mandatory.[14]

In 1982, a working formulation of NHL was introduced categorizing the NHLs into low grade, intermediate grade, high grade and miscellaneous. This classification was welcomed by the clinicians and was in use for a long time since this way of classifying lymphomas also indicates the prognosis.

The advent of immunological methods encouraged the classification of lymphomas based upon the cell type. An updated Kiel classification was

introduced in 1989 and was followed in many European countries, though the Americans retained the use of working formulation for classifying lymphomas. It classified NHL into B and T cell lymphomas, which in turn were again classified into low grade, high grade and rare tumors.[14]

In 1994, an international lymphoma study group proposed the Revised European American classification of lymphomas (REAL) in order to achieve consensus between the European and American nations. It took into consideration the morphologic, immunologic and genetic characteristics of the lymphomas.[13] The lymphomas were classified as B cell neoplasms, T cell neoplasms and Hodgkin disease. The B and T cell neoplasms were again classified into precursor neoplasms and peripheral neoplasms.

WHO classification of the hematopoietic and lymphoid neoplasms proposed in the year 2003 took into consideration all the classifications used in various parts of the world and sought the opinion of renowned clinicians across the globe. This was the first classification to be agreed throughout the globe. The current WHO classification is a revision of the 2003 classification. It provides a separate category for lymphomas which cannot be classified under a specific category, thereby making it easy to recognize the cases which require further study.

The current WHO classification of hematopoietic and lymphoid neoplasms, 2008 classifies lymphomas under the following 6 broad groups: [3]

- ❖ Precursor lymphoid neoplasm
- ❖ Mature B cell neoplasms.
- ❖ Mature T cell and natural killer (NK) cell neoplasms
- ❖ Hodgkin lymphoma
- ❖ Histiocytic and dendritic cell neoplasm
- ❖ Post transplant lymphoproliferative disorders.

The following table no :2 gives the detailed classification of both NHL and HL adopted by WHO in 2008.

TABLE 2: WHO CLASSIFICATION OF LYMPHOMAS 2008

PRECURSOR LYMPHOID NEOPLASMS	• B lymphoblastic lymphoma, NOS • B lymphoblastic lymphoma with recurrent genetic abnormalities • B lymphoblastic lymphoma with t(9;22) • B lymphoblastic lymphoma with MLL rearranged • B lymphoblastic lymphoma with t(12;21) • B lymphoblastic lymphoma with hyperdiploidy • B lymphoblastic lymphoma with hypodiploidy • B lymphoblastic lymphoma with t(5;14) • B lymphoblastic lymphoma with t(1;19) • T lymphoblastic lymphoma

MATURE B CELL NEOPLASMS	• Small lymphocytic lymphoma (SLL/CLL) • B cell prolymphocytic leukemia • Splenic B cell marginal zone lymphoma • Hairy cell leukemia • Splenic B cell lymphoma • Lymphoplasmacytic lymphoma (LPL) • Heavy chain diseases • Plasma cell myeloma • Solitary plasmacytoma of bone • Extra-osseous plasmacytoma • Extranodal marginal zone lymphoma of mucosa associated lymphoid tissue (MALT lymphoma) • Nodal marginal zone lymphoma (MZL) • Follicular lymphoma (FL) • Primary cutaneous follicle centre lymphoma • Mantle cell lymphoma • Diffuse large B cell lymphoma (DLBCL) • DLBCL associated with chronic inflammation • Lymphomatoid granulomatosis • Primary mediastinal large B cell lymphoma • Burkitt lymphoma • B cell lymphoma , unclassifiable,with feature intermediate between DLBCL and Burkitt lymphoma • B cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical HL

MATURE T CELL AND NK CELL NEOPLASMS	• T cell prolymphocytic lymphoma • T cell large granular lymphocytic lymphoma • Aggressive NK cell leukemia • Systemic EBV positive T cell lymphoproliferative disease of childhood • Hydroa vacciniforme like lymphoma • Adult T cell lymphoma • Extranodal T cell lymphoma, nasal type • Enteropathy associated T cell lymphoma • Hepatosplenic T cell lymphoma • Subcutaneous panniculitis like T cell lymphoma • Mycosis fungoides • Sezary syndrome • Primary cutaneous CD30 positive T cell lymphoproliferative disorders • Lymphomatoid papulosis • Primary cutaneous peripheral T cell lymphomas, rare types • Primary T cell lymphoma, NOS • Angioimmunoblastic T cell lymphoma • Anaplastic large cell lymphoma, ALK positive • Anaplastic large cell lymphoma, ALK negative
HODGKIN LYMPHOMA	(Classified as described earlier)

HISTIOCYTIC AND DENDRITIC NEOPLASMS	• Histiocytic sarcoma • Langerhans cell histiocytosis • Langerhans cell sarcoma • Interdigitating dendritic cell sarcoma • Follicular dendritic cell sarcoma • Fibroblastic reticular cell tumor • Indeterminate dendritic cell tumor • Disseminated juvenile xanthogranuloma
POST TRANSPLANT LYMPHOPROLIFERATIVE DISORDER (PTLD)	• Polymorphic PTLD • Monomorphic PTLD • Classical HL type PTLD • Plasmacytic hyperplasia and infectious mononucleosis like PTLD

Once a lymphoma is diagnosed and is classified under a specific category, it needs to be staged like any other neoplasm. The Ann-Arbor staging system has been in use for staging both the HL and NHL.[1][11] In addition to the history of illness, physical examination and radiological data are essential for staging the disease. A minimum of ultra sonogram of abdomen has to be done in order to stage the disease efficiently. [14] The staging system stratifies lymphomas into four stages, from stage I to stage IV. A letter 'E' is added to this stage in case the primary tumor is in an extra-lymphoid organ, which are the organs other than lymph node, spleen, thymus, Waldeyer's ring, appendix and Peyer's patches. These stages are again sub-classified based upon the presence (B) or absence (A) of systemic symptoms like

- ❖ Unexplained fever
- ❖ Unexplained weight loss of more than 10% of body weight in preceding 6 months
- ❖ Night sweats

The following table 3 illustrates the Ann-Arbor staging.[1]

TABLE 3: ANN – ARBOR STAGING FOR LYMPHOMAS

STAGE I	**Involvment of a single lymph node or a single extralymphoid organ**
STAGE II	Involvement of two or more lymph node regions on the same side of the diaphragm or localized involvement of an extralymphatic organ and one or more lymph node regions on the same side of the diaphragm
STAGE III	Involvement of lymph node regions on both side of the diaphragm, which may also be accompanied by localised involvement of an extralymphatic organ or involvement of spleen or both
STAGE IV	Diffuse or disseminated involvement of one or more extralymphatic organs or tissues with or without associated lymph node enlargement
SUBCLASSIFICATION: **A- Without the symptoms listed below** **B- With systemic symptoms** ❖ **Unexplained fever** ❖ **Unexplained weight loss of more than 10% of body weight in preceding 6 months** ❖ **Night sweats**	

The accurate diagnosis of lymphoma can only be made with adequate clinical history. Some of the lymphomas have certain clinical findings reserved to them and aid in the diagnosis. The important clinical findings that are essential in the diagnosis of lymphoma include

- Age
- Sex
- Site
- Duration
- Presence of B symptoms
- Presence of lymphadenopathy
- Associated organomegaly
- History of treatment
- Skin lesions
- History of associated infections like HIV, HBV, HCV etc.

The clinical picture not only drives a pathologist towards a diagnosis but also helps the pathologist to assess the optimal ancillary technique that will be required in a particular case.

HISTOLOGY OF A NORMAL LYMPHNODE:

A thorough knowledge about the histology of the lymph node is mandatory before assessing a case of lymphoma. This is because certain lymphomas have characteristic pattern of involving the lymph node and the lymphomas as such are presumed to resemble the normal counterparts of the cells in

lymph node, and hence named after them.[15] For example marginal zone lymphoma and mantle cell lymphoma are named so because of their resemblance to marginal zone cells and mantle cells of a normal lymph node respectively. Knowing the histology of lymph node also helps in understanding the pattern of immunohistochemical markers in a normal lymph node.

Lymph nodes are encapsulated, bean shaped, firm structures, whose size vary with site and activity. The afferent lymphatics enter through the convex surface and the efferent lymphatics and veins leave through the hilum. Ateries and nerves enter through the hilum. The lymph node is composed mainly of three regions, which are:[16][17]

- Cortex
- Medulla
- Paracortex

The cortex is identified by the presence of lymphoid follicles with cortical sinuses running between them. The follicles can be primary follicles or secondary follicles. The secondary follicles have a germinal centre, surrounded by a mantle zone which in turn is surrounded by a marginal zone. Follicular dendritic cells and tingible body macrophages are present in the germinal centre in addition to the lymphoid cells. The lymphoid cells are predominantly B cells admixed with a few T cells. The B cells in the follicles are either centroblasts or centrocytes. Centroblasts have a vesicular nuclei

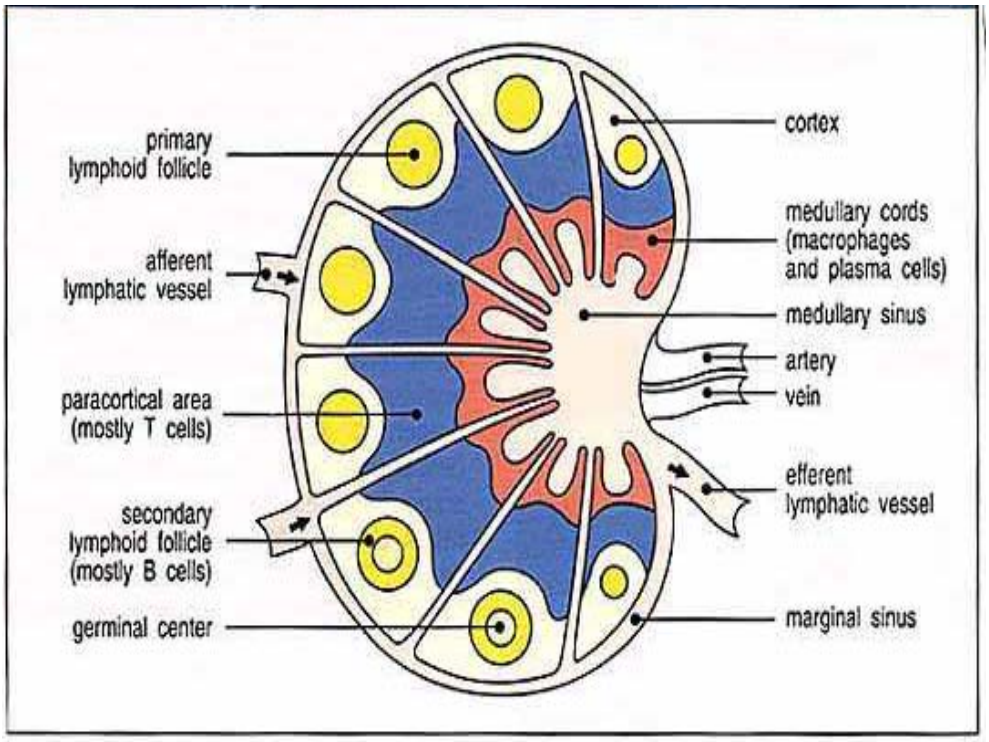
and amphophilic cytoplasm whereas centrocytes have a cleaved nuclei, dense chromatin and scant cytoplasm. The cells of the primary follicles and the mantle zone are small and have dense chromatin and the cells in the marginal zone are similar but slightly larger and have more cytoplasm. [16][17]

The medulla is made up of cords of cells, mainly plasma cells with intervening medullary sinuses which contain histiocytes.

The region between the cortex and medulla is called paracortex. This region is highly vascular and is rich in T lymphocytes. [16][17]

We need to bear in mind that the architecture of the lymph node is not static and it changes with different stimuli. The lympnodes from different sites show different morphology. For example the abdominal nodes show a prominent marginal zone. This should not be confused with a reactive or neoplastic process. The following figure (fig:1) depicts the structure of a lymph node as in google images site//www.dartmouth.edu/... in the article overview of the lymphoid immune system[18]

Figure 1: STRUCTURE OF A LYMPHNODE



MORPHOLOGICAL APPROACH TO LYMPHOMA:

In almost all the cases of nodal lymphomas, the normal structure of the lymph node is completely or partly replaced with the neoplastic cells. Some of the lymphomas like follicular lymphoma (FL) do not disturb the normal structure of a lymph node and hence can be confused with a reactive process. FL can be differentiated from a reactive follicular hyperplasia by the presence of prominent mantle zone. Viral infections can show numerous immunoblasts that can be confused with RS cells. Histiocyte rich areas in Kikuchi's lymphadenitis and necrotic areas can lead to considering a DLBCL in the

differential diagnosis. Therefore we need to bear in mind all the reactive conditions that simulate a lymphoma before making a diagnosis of lymphoma.[1]

Once all the reactive conditions have been ruled out, the pattern of the lymphoma needs to be assessed. Different lymphomas have a different pattern of involvement and are characteristic to the type of lymphoma. The commonly seen patterns in a lymphoma include

- Nodular
- Mantle zone
- Marginal zone
- Sinusoidal
- Starry sky
- Interfollicular
- Vascular
- Diffuse

Nodular pattern of involvement is seen in cases like follicular lymphoma and nodular type of mantle cell lymphoma or marginal zone lymphoma. NLPHL and nodular sclerosis type of classical HL also show a nodular pattern. These nodules are separated by dense fibrous tissue. Sometimes ALCL also has this pattern of involvement. The presence of RS cells or L and H cells aid in its diagnosis of HL. The nodules formed by small lymphocytic lymphoma and

rarely by lymphoplasmacytoid lymphoma are vague and are also called pseudonodular or pseudofollicular pattern.[1]

Mantle zone and marginal zone pattern are seen in mantle cell lymphoma and Marginal zone lymphoma repectively. Starry sky pattern, called so due to the presence of numerous tingible body macrophages interspersed with them is characteristic feature in Burkitt's lymphoma. This pattern is also noted in lymphoblastic lymphoma.[1]

Lymphomas like ALCL, mycosis fungoides etc can be seen involving only the sinuses and spread along the sinuses. Carcinomas metastasizing to the lymphnodes also involve the sinuses first. Hence care should be taken to avoid misdiagnosis. An interfollicular pattern of involvement is seen commonly in the T cell lymphomas. Some lymphomas like have a prominent vascular proliferation. This vascular pattern in commonly seen in angioimmunoblastic T cell lymphomas and peripheral T cell lymphomas. A diffuse pattern of involvement is seen in almost all types of lymphomas. The following table (table 4) shows the common pattern of involvement seen in various lymphomas

TABLE 4: VARIOUS PATTERNS AND TYPES OF LYMPHOMA

PATTERN	LYMPHOMA
Nodular	• Follicular lymphoma • Mantle cell lymphoma • Marginal zone lymphoma • NLPHL • Nodular sclerosis HL • Small lymphocytic lymphoma • Lymphoplasmacytoid lymphoma • Anaplastic large cell lymphoma
Mantle zone	Mantle cell lymphoma
Marginal zone	Marginal zone lymphoma
Starry sky pattern	Burkitt's lymphoma Lymphoblastic lymphoma
Sinus pattern	Anaplastic large cell lymphoma Mycoises fungoides Marginal zone lymphoma
Interfollicular pattern	T cell lymphomas
Vascular pattern	Angioimmunoblastic T cell lymphoma Peripheral T cell lymphoma NOS

After identifying the pattern, the morphology of individual cells needs to be examined, starting from the size of the cells. The cells can be divided into three types:

- ❖ Small cells

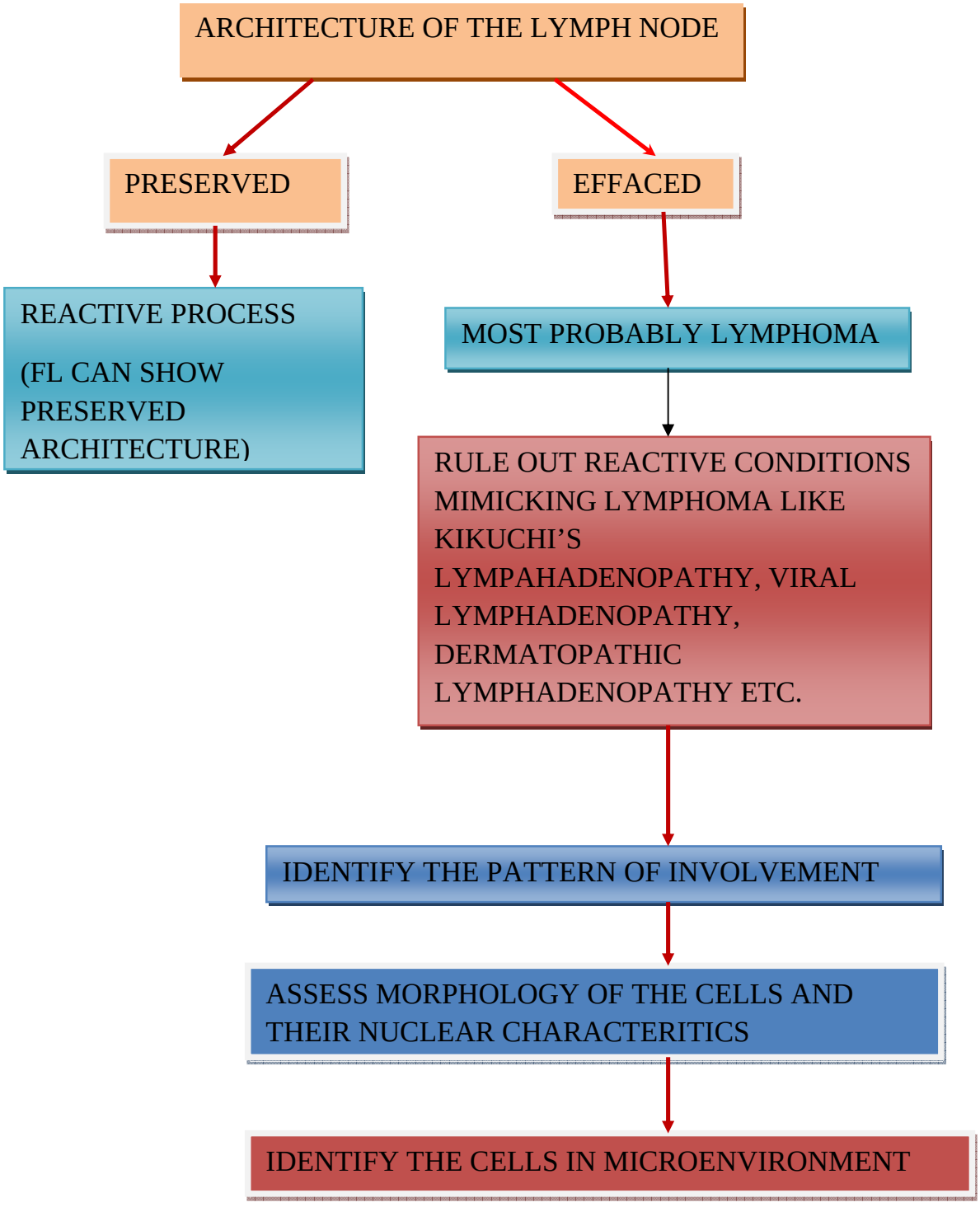
- Medium sized cells
- Large cells

The cells are compared with the size of the endothelial cells. The cells smaller than endothelial cells are called small cells and those larger than them are called large cells. The cells which are just about the size of the endothelial cells are called medium sized cells. Other fine details like the shape of the nucleus and chromatin pattern are also observed. The small cells are seen commonly in follicular lymphoma and mantle cell lymphoma while the large cell morphology is seen in ALCL, DLBCL and plasmablastic lymphoma.

The neoplastic cells can be admixed with a reactive cell population. Identifying the reactive population helps in the diagnosis. These cells in the background form the basis for classifying HL.[1]

The following figure 2 summarises the approach to a case of lymphoma in the routine H and E slide.

FIGURE 2: ALGORITHM OF APPROACH TO A CASE OF LYMPHOMA



IMMUNOHISTOCHEMISTRY:

Immunohistochemistry is a method of identification of the cellular constituents with the help of antigen antibody reaction.[19] It is based on the principles used for immunofluorescence, but does not require a specialized microscope for assessing the antigen antibody reaction. Antigens labelled with enzymes which produce a colourful reaction on addition of a chromogen is used in place of a fluorescent dye labelled antibodies in immunofluorescence.

Flow cytometry is another technique that can be used for immunophenotypic analysis. But it requires sophisticated machinery and fresh specimen. Though IHC was initially practised on fresh and frozen specimens, with the advent of antigen reviving techniques and development of antibodies which can be used on paraffin embedded tissues, IHC can now be reliably practised on routine paraffin sections.[20][21][22] The morphology of the cells cannot be assessed in flow cytometry. IHC has the added advantage of morphological analysis and immunological analysis simultaneously. Hence IHC is superior to flow cytometry in certain conditions, especially lymphomas.[20] In addition, IHC facilitates the identification of the antigen antibody reaction at the sub cellular level i.e. the membrane, nucleus, Golgi apparatus etc.[19]

The IHC markers can be categorized into three types, based upon their use as follows:

- ❖ Diagnostic markers
- ❖ Predictive markers
- ❖ Prognostic markers

Diagnostic markers are those which help in making diagnosis, like in case of a poorly differentiated tumour and in differentiating various other neoplasms from the normal or reactive process. Predictive markers are those which help in assessing the response to an available treatment. They predict the outcome of a therapeutic method and hence they are called so. Prognostic markers are those markers which help in determining the aggressiveness of the neoplasm and patient's survival.[23]

The majority of IHC markers used in lymphomas are for the leucocyte differentiation antigen and have an acronym CD which stands for 'cluster of differentiation'. It was standardised to use this acronym followed by a number that corresponds to the small region, called epitope on the antigen, in order to overcome the confusion arising due to the different names used for the same marker by different investigators. The monoclonal antibodies are directed against the epitopes.[20]

VALUE OF IHC IN LYMPHOMAS:

Diagnosing a case of lymphoma and subtyping the lymphoma is one of the most challenging tasks for a pathologist. Lymphoma can be easily confused with other nonlymphoid neoplasms like poorly differentiated carcinomas, small cell/ neuroendocrine carcinomas, peripheral neuroectodermal tumors

(PNET), desmoplastic small round cell tumor (DSRCT), rhabdomyosarcoma, Wilms tumor etc. A positive reaction with CD45, also known as leucocyte common antigen helps in establishing the diagnosis as lymphoma and rules out the other possibilities.[19]

There is a myriad of reactive conditions which mimic the lymphomas. IHC plays an important role in differentiating reactive process from a lymphoma. Even cases of HL can be confused with reactive node when the diagnostic Reed-Sternberg cells are scarce. IHC staining with CD15 and CD30 can help in establishing the diagnosis. The pattern of IHC staining is also helpful to differentiate the lymphomas from a reactive process. For example in a case of follicular lymphoma, Bcl-2 is negative in the follicular centre, whereas it is positive in a reactive lymph node.

Once a diagnosis of lymphoma is made, it needs to be categorised as HL or NHL, followed by placing it under a particular subtype. Morphology in adjunct with IHC is sufficient to type most of the lymphomas. The Non Hodgkin's lymphoma project observed a significant increase in accurate diagnosis and sub-classification of lymphoma using IHC.[24] Some of the lymphomas have characteristic immunophenotypes that the presence of such immunophenotype is essential for making that diagnosis.

Though HL can be diagnosed with the presence of Reed-Sternberg cell, it can be confused with diffuse large B cell lymphoma (DLBCL) when they are abundant. A uniform strong positivity with CD20 in DLBCL helps in

distinguishing them from HL. Nodular lymphocyte predominant Hodgkins lymphoma (NLPHL) with a predominant T cell population in the reactive component brings T cell rich B cell lymphoma into the differential diagnosis. In such cases, CD57+ve, CD4+ve T cell rimming around the Lymphohistiocytic cell (L H cell) helps in recognising them as NLPHL. Anaplastic large cell lymphoma (ALCL) is another lymphoma having cell that resemble the RS cells, but these cases are positive for ALK, clusterin and fascin. Certain antigens are preserved even in some necrotic tissues and IHC can be helpful in detecting these antigens in biopsies containing large areas of necrosis.[25]

In case of NHL, the lineage of the lymphoma needs to be recognised. IHC markers are essential for establishing the lineage. CD20, CD79a, CD19 etc are commonly used for determining B cell lineage. All these markers can be used on paraffin embedded sections except CD19. The clonality of the B cell neoplasm can be established with the use of immunohistochemical staining with kappa and lambda light chains. CD3 is the frequently used pan T cell marker. [25]

Further characterisation of the neoplastic cells may be required after the recognition of the lineage. In case of B cell lymphomas CD5, CD10, CD23, BCL2 and BCL6 have been used and in case of T cell lymphomas CD2.CD4, CD5, CD7 and CD8 are used for this purpose. The absence of staining with pan T cell marker and altered CD4:CD8 ratio suggests a T cell lymphoma. [25]

The following figures (figure3 and 4) shows the use of IHC markers in diagnosing lymphomas and sub-categorizing them.

FIGURE 3: Role of IHC markers in subtyping Hodgkin lymphomas

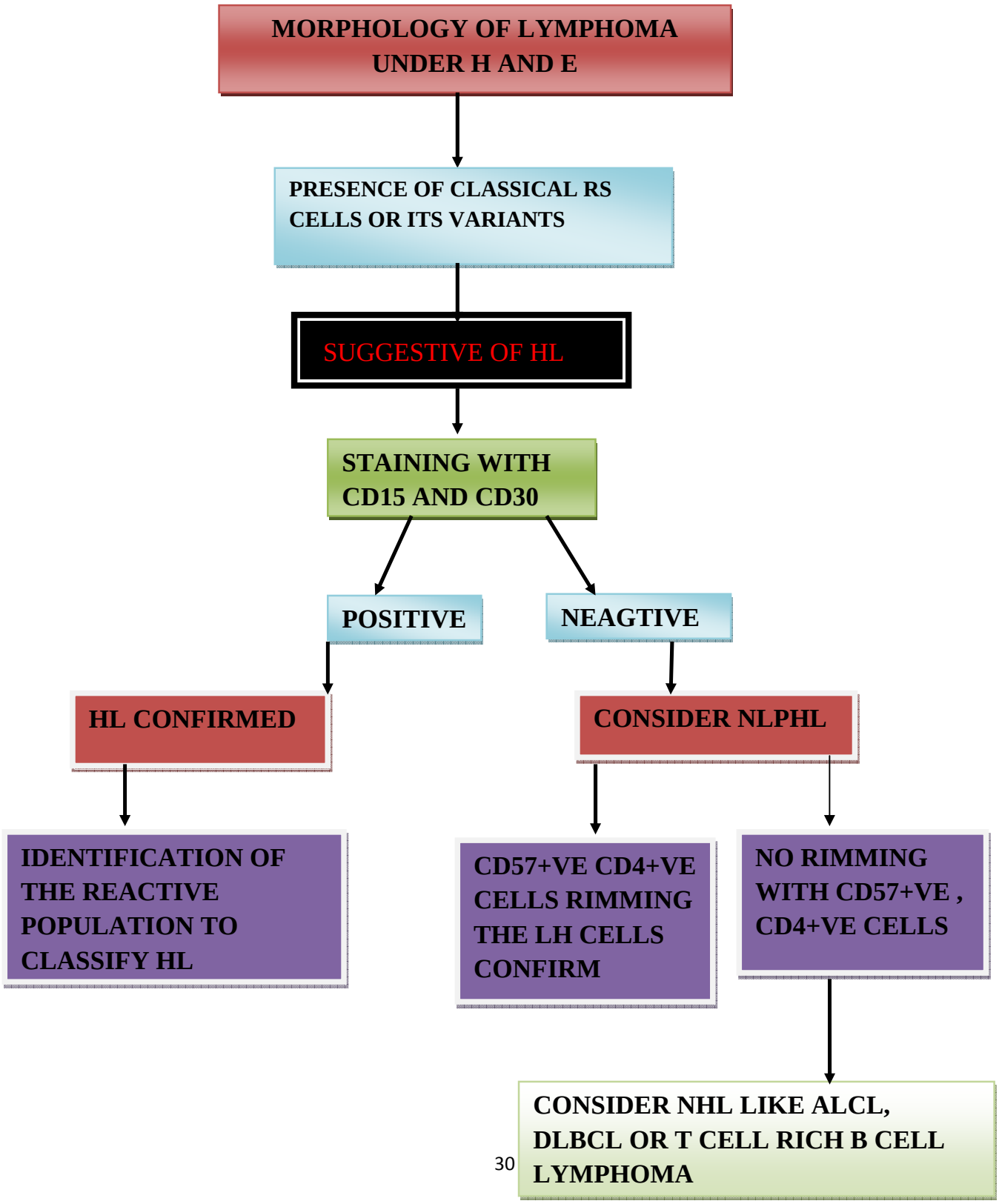
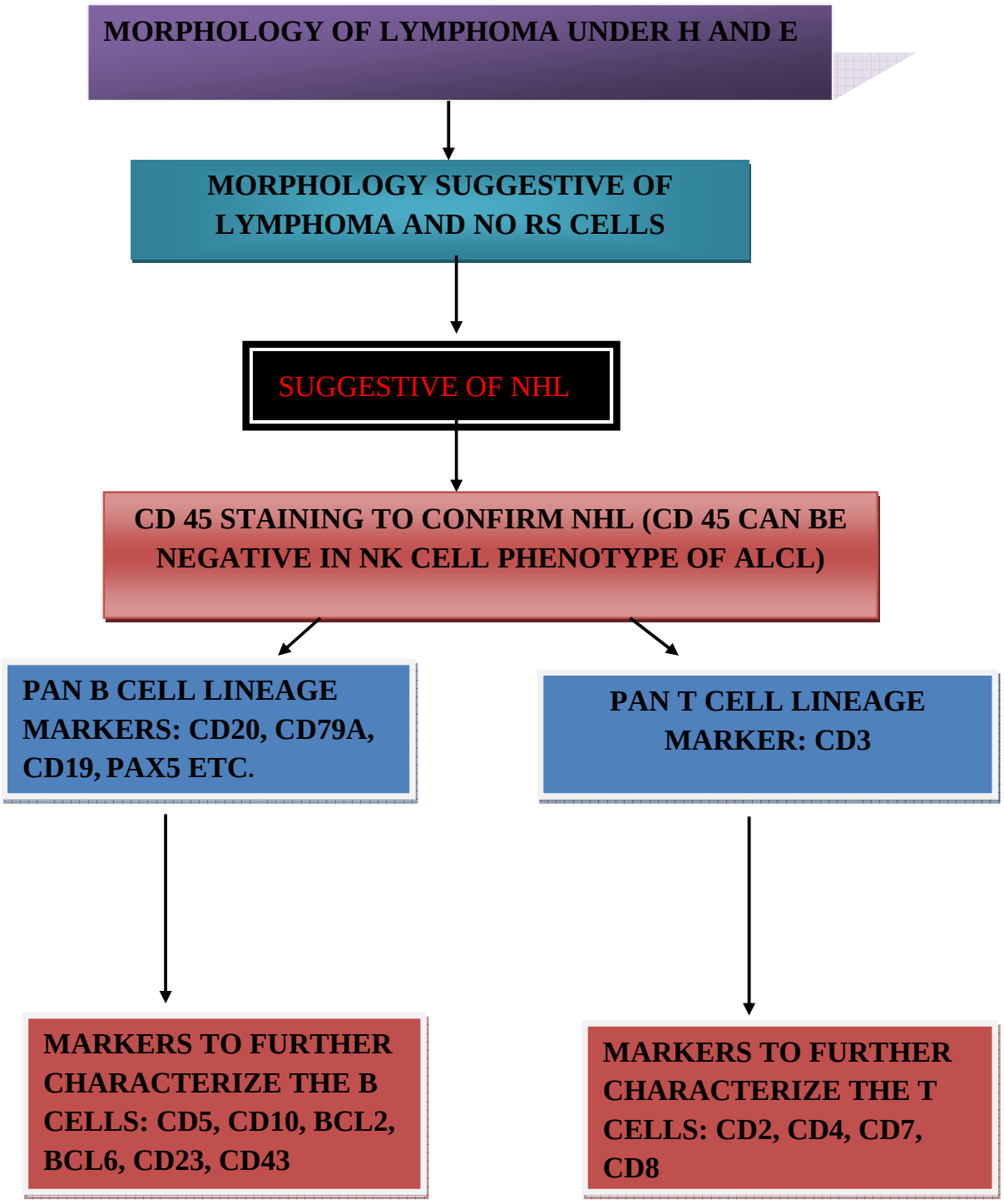


FIGURE 4: Role of IHC markers in subtyping non Hodgkin lymphomas



IHC markers can also be used to assess the prognosis in lymphomas. DLBCL cases showing positive reaction with Bcl2, MUM1, FOXP1 and negative reaction with CD10 were found to have a bad prognosis. ALK positive cases of ALCL carry a good prognosis. Ki67/ MIB1 staining shows the proliferation index.[23] Lymphomas with high proliferation index have a bad prognosis. Reinhard Von Wasilewski et al observed that cases of HL that lack the expression of CD15 showed a bad prognosis.[26]

The identification of the cell antigens has become more important ever since the successful introduction of Rituximab in the treatment of lymphomas. Rituximab is an anti CD20 therapy targeted against the cells having this surface antigen. The success of Rituximab has driven the investigators to identify other therapeutic targets. The drugs targeting other cell antigens like CD22, CD30, CD40, CD80 are still in the research process.[23]

Thus immunophenotypic analysis in addition to being an integral part of WHO classification is also essential in making the correct diagnosis and assessing the prognosis. Though IHC plays a vital role in diagnosing lymphomas, morphological assessment still remains essential. IHC markers should be used cautiously and judiciously. A single marker is never sufficient in a case of lymphoma. Hence a panel of markers is almost always necessary.[25] The panel of markers commonly used in a case of lymphoma include:

- CD45 – to confirm the diagnosis of lymphoma (CD 45 is negative in classical HL and NK cell phenotype of ALCL)
- CD3 – pan T cell marker
- CD20- pan B cell marker
- CD15 and CD30 – for diagnosing HL.

CD45:

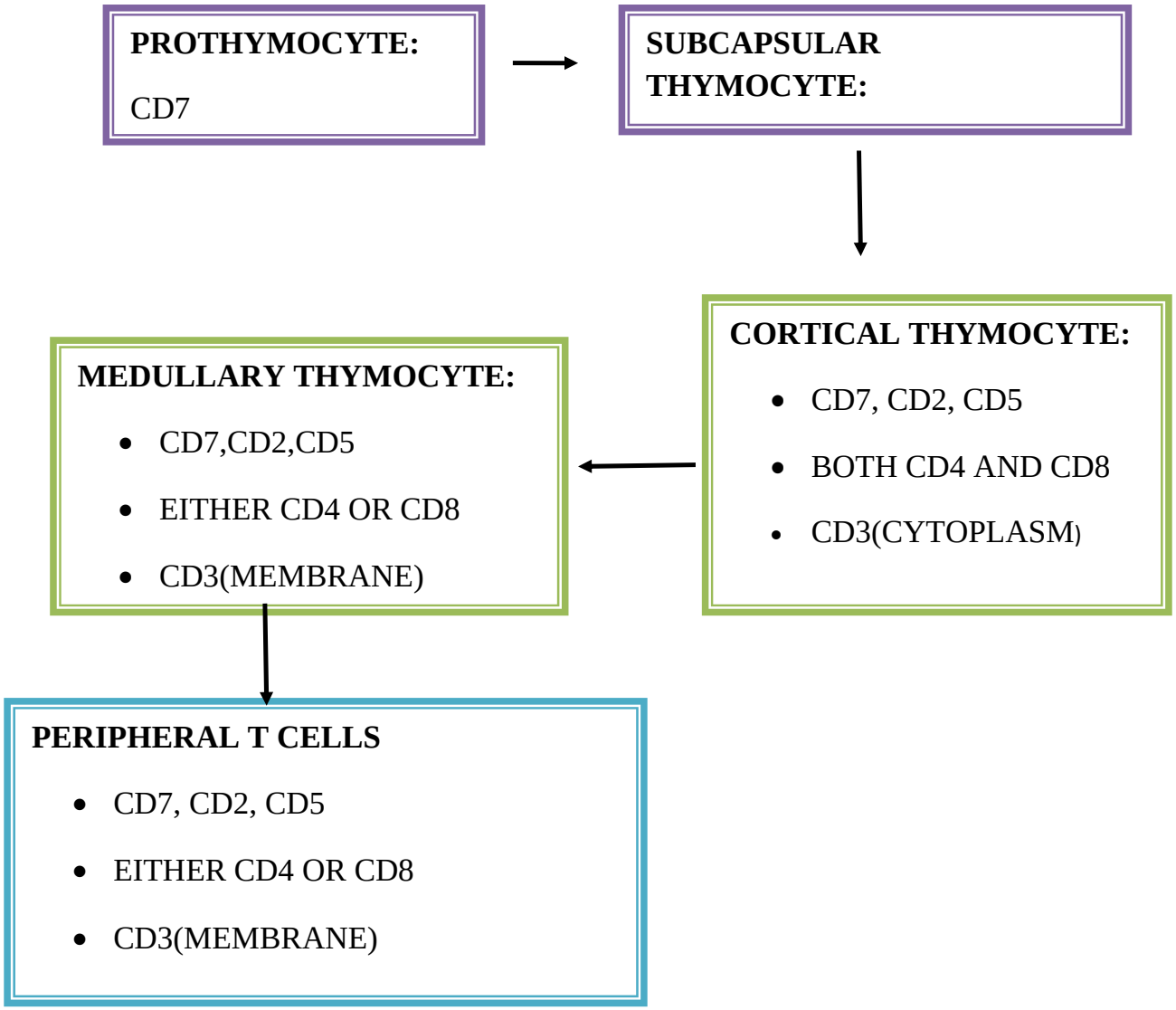
CD45, also referred to as leucocyte common antigen (LCA) is a tyrosine phosphatase present on the surface of the leucocytes. CD45 is lost in plasma cells.[27] This antigen is rarely present in the Golgi apparatus in addition to the cell membrane. Some isoforms of CD45 are specific to certain cells. For example CD45RO is specific for T cell, histiocytes and CD45RA is seen predominantly in T cells.[1][27] CD45RB is the commonly used isoform as it is present in all leucocytes. Its primary role is to identify a lymphoma and to differentiate it from other tumors resembling lymphoma. CD45 expression is lost in classical HL and in cases of ALCL with NK cell phenotype. [1]

CD3:

CD3 is also referred by different names like Leu 4, T3 etc... [21] It is the T cell receptor protein complex composed of gamma, delta and epsilon chains. CD3 is initially positive in the cytoplasm and is transferred to the cell membrane with maturation. CD3 is negative in NK cells, though a cytoplasmic positivity can be

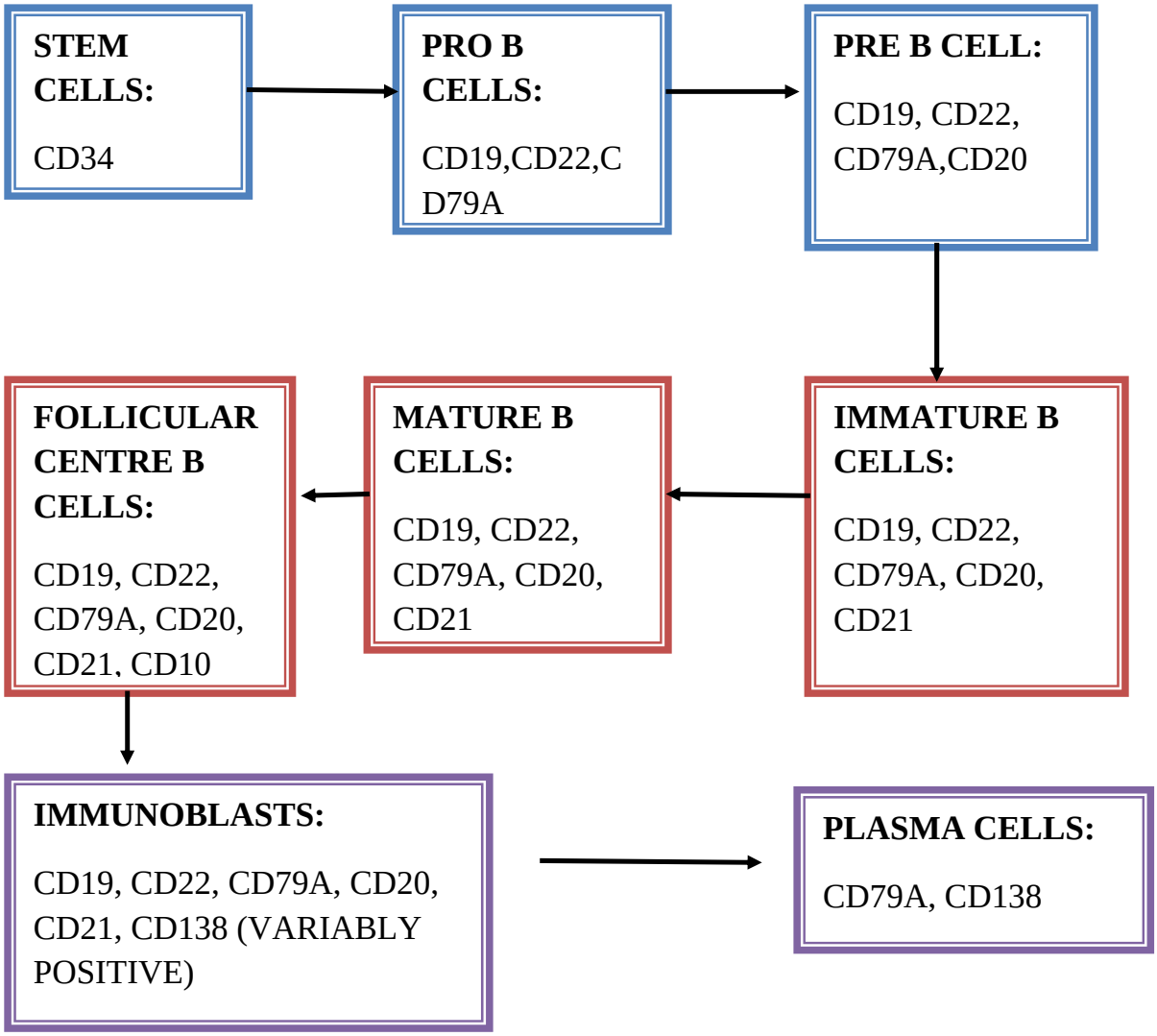
seen in some NK cells. [15] CD 3 is preferred over other T cell markers because of its specificity and easy, reliable detection on paraffin embedded sections.[27] The following figure 5 illustrates the various CD markers expressed during various stages of maturation of T cells.

Figure 5: T cell development with the respective immune profile



CD20 is also known by other names like Leu26 and B1.[21] It is a nonglycosylated phosphoprotein seen on cell membrane surface of all the mature B cells. Its expression is seen from the pre B cell stage of maturation and is lost in plasma cell stage. The following flow chart shows the expression of various CD markers during the development of B cells.

Figure 6: B cell development with the respective immuomarker profile



CD20 is expressed in all the mature B cell lymphomas. It is expressed in a part of classical HL and is seen in all NLPHL.[12][28] Rare CD20 expression is seen in some T cell lymphomas

CD15:

It is also referred to as Lewis X antigen, X hapten, Leu M1 and myelomonocytic marker.[21][27] It is a cell adhesion molecule and its expression is seen on the cell membrane of RS cells of classical HL with or without dot like golgi localization.[27] CD15 is positive only in a part of HL and its expression is believed to have a better prognosis. Certain B and T cell lymphomas may also be positive for CD15 and ALCL is always negative for CD15. Its expression is confined to the lymphomas and is noted in other cells like granulocytes, histiocytes and other non hematolymphoid tissues like breast, proximal convoluted tubules of kidney, lung and Paneth cells in GI tract.[29]

CD30:

CD30 is also referred to as Kil, Ber H2 and lymphocyte activation antigen.[21] It belongs to TNF receptor superfamily and is always positive in classical HL and ALCL. CD30 mediated NFkB activation is believed to be the pathophysiology behind classical HL.[30][31] It is seen as a membranous positivity with or without dot like golgi localization. The followingtable (table 5) summarizes the

expression of the commonly used CD markers in various situations and the subcellular site of positivity.

TABLE 5: CD MARKERS WITH THE STAINING PATTERN AND POSITIVE CELLS

MARKERS	SITE OF POSITIVITY	COMMONLY POSITIVE CELLS
CD45	Membrane	All leucocytes except plasma cells
CD3	Membrane	T cells
CD20	Membrane	B cells
CD15	Membrane with or without golgi positivity	RS cells, activated T lymphocytes , proximal convoluted tubules of kidney, Paneth cells, tumours of lung, breast , thyroid and GIT
CD30	Membrane with or without golgi positivity	RS cells, ALCL, activated B cells and monocytes

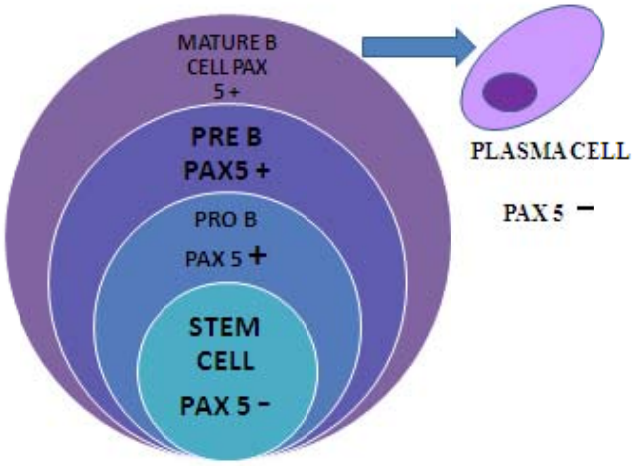
In addition to the available wide range of CD markers for determining the lineage of lymphomas, there are other markers which are specific for the lineage. PAX5 is one such marker which is expressed exclusively in B cells.

PAX5:

PAX stands for paired box. The PAX proteins are all transcription factors that usually determine the fate of the cells during the early stages of development and maturation mostly during embryogenesis and sometimes even in the adult life. They are closely related proteins and are called paired box as they have a paired domain for binding to the DNA .Genes encoding the PAX proteins are called PAX genes. There are about nine PAX genes identified till date.[4]

PAX5 is otherwise called B cell specific activator protein (BSAP). It is named so because of its exclusive expression in B lymphoid lineage. It is a transcription protein like other PAX proteins and its expression is noted as early as pro B cell stage and is lost during the plasma cell stage.[4] (Figure 7) PAX5 drives the cells into B cell lineage and hence plays an essential part in B cell development. The genes that encode the PAX5 is situated in the chromosome 9p31.

FIGURE 7: B CELL MATURATION AND PAX 5 EXPRESSION



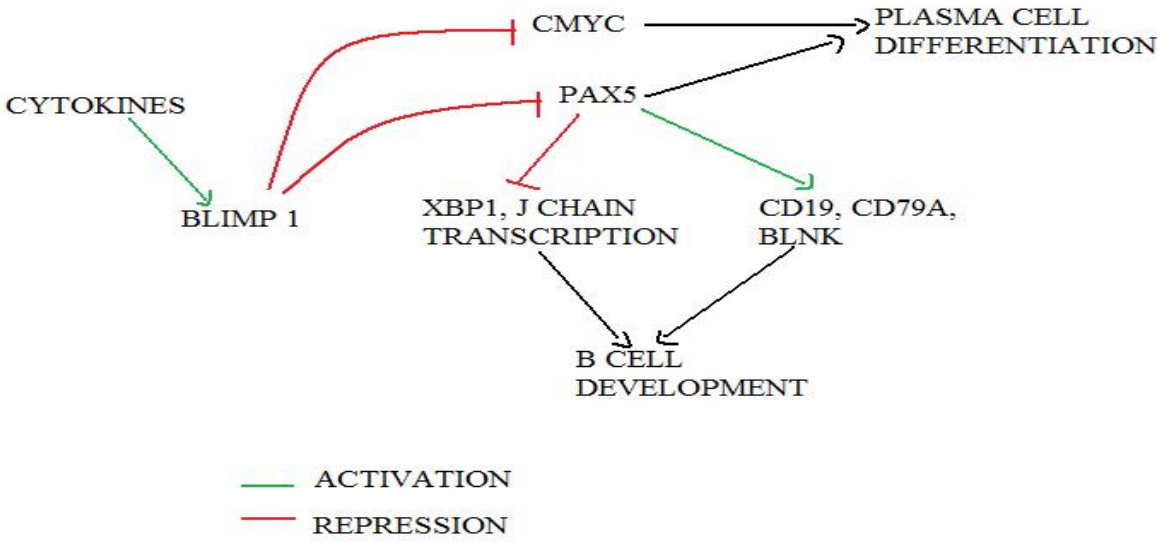
The role of PAX5 in B cell differentiation and maturation is mainly due to its ability to regulate the CD19 gene. CD19 is a trans-membrane protein involved in the transduction of signals from B cell receptors that are specific to B cells. CD19 is involved in B cell proliferation in addition to its functions in regard to the immune system.[32] Thus by regulating the expression of CD19, PAX5 regulates the generation and maturation of the B cells. The other genes that are activated by PAX5 include:

- CD79a
- B lymphoid kinase (Blk)

PAX5 suppresses the J chain and XBP1, thereby preventing the cells from maturing into plasma cells. Thus BSAP is not only essential in driving the cells towards B cell lineage, but also retaining its identity. [33] PAX5 along with PAX2 and PAX8 has been demonstrated to influence the apoptosis and aid in survival of the cell in nematodes. Hence they are believed to have a role in survival of the B cells. [34]

The suppression of PAX5 is mandatory for plasma cell differentiation. This is achieved through increased expression of B lymphocyte induced maturation protein 1 (Blimp 1), which is induced by various cytokines. Blimp1 suppress the expression of PAX5 in addition to other genes like C-myc. [33] Following diagram (fig 8) illustrates the role of PAX5 in B cell development and the mechanism by which BLIMP 1 drives the cells towards plasma cell differentiation

Figure 8: The role of PAX5 in B cell development and the mechanism by which BLIMP 1 drives the cells towards plasma cell differentiation



Though immunohistochemical expression of PAX5 is noted in all stages of maturation the intensity of staining varies in subsets of B cells. Krenacs et al observed a strong PAX5 immunoreactivity in the cells of the marginal zone as opposed to the follicular centre cells and monocytoid B cells. [35]

PAX5, in addition to the B lymphocytes is also observed in CNS during the early stages of maturation and in adult testis. [4] In contrast to these findings, Tarlakovic et al observed an absent immunohistochemical reaction of PAX5 in adult testis and a positive immunoreactivity in certain parts of the adult brain tissue like gray matter of mid brain, neurons of the area postrema, medulla

oblongata and a few cells in the caudal nucleus. PAX5 is strongly expressed in mesonephric rests of epididymis and smooth muscle cells of uterus. Rare cells in prostatic glands and endocervical glands express PAX5 weakly. [36] Thus in addition to B cell development and CNS development, PAX5 is thought to play a role in urogenital development.

The various types of B cell lymphomas are believed to represent the cells of different stages of maturation.[15][23] PAX 5 expression is noted in majority of mature B cell lymphomas, premature B cell lymphomas and HL. They show a sharp nuclear reactivity making the assessment of immunoreactivity easy since they are devoid of background staining. Being a nuclear antigen, their immunoexpression is altered with poor tissue preservation. [5]

The main utility of PAX5 is its expression in B cell lymphomas which lack or show equivocal immunoreactions with the commonly used IHC markers in paraffin embedded sections like CD20. CD20 expression is usually absent in classic HL and precursor lymphoid neoplasms. The surface CD20 expression is lost in patients following Rituximab therapy as it is a targeted therapy against CD20. In case of relapse in these patients, the cells may remain to be negative for CD20. PAX 5 helps in situations like these to establish the cell lineage.

Though PAX5 expression is seen in most of the cases of both classical HL and NLPHL, the intensity of staining is weak in a majority of the cases. Some cases of HL can be negative for PAX5 especially in cases of nodular sclerosis type of

classical HL. This reduced expression is due to the down regulation of PAX5 along with other transcription factors and surface antigens. Blimp1 has been observed to be expressed in certain classical HL. This is thought to be the reason behind the reduced expression of PAX5 in some cases and even for the absence of PAX5 reaction in some cases.[5]

The expression of PAX5 is absent in a few but a significant number of cases of lymphomas with large cell morphology like DLBCL. A study by Torlakovic et al showed PAX5 was positive in about 96% of cases, of which 90% showed strong positivity. These findings correlated with the immunoexpression of CD20. [38] Therefore though, PAX5 is negative in few cases of DLBCL, it is equivalent to CD20 in its ability to recognize DLBCL. The weak or absent immunoreativity with PAX5 in DLBCL may be because they represent the post follicular cells in normal B cell development. [35]

T cell lymphomas are consistently negative for PAX5. Its expression in T cell lymphomas was observed very rarely by different authors.[39][40][41] These cases are thought to represent the aberrant or over expression of PAX5 in these tumors and Feldman et al observed extra copies of the PAX5 genes in such cases. Over expression of PAX5 is observed to induce T cell lymphomas in experimental animals. Hence PAX5 was postulated in tumorigenesis of these cases.

Problems can arise in differentiating between DLBCL, ALCL and HL as all these lymphomas can show large cells closely resembling RS cells. The routinely used IHC markers can add on to the confusion as these tumors can have similar immunophenotypes. Browne et al used PAX5 along with other B cell transcription factors oct2, BOB1 and other pan B cell antigens in known cases of ALCL, DLBCL and HL. PAX5 was observed to be negative in all the cases of ALCL and positive in all cases of DLBCL and NLPHL. It was positive in about 91% of classical HL.[42] The negative reaction of PAX5 in T cell lymphomas can be exploited in situations like these.

The expression of PAX5 has also been observed in acute leukemia, both acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL). PAX5 positive cases of AML mostly belonged to AML with t (8: 12) subtype, but cases of AML belonging to other subtypes also showed positive reaction in a few instances. AML with t (8:12) is known to express CD19, which is regulated by PAX5 and hence its expression is these cases. The number of PAX5 positive cases in this subtype of AML varied among different studies. Sarah E Gibson et al observed PAX5 being expressed in about 44% of this subtype of AML, [43] whereas Joe R Vulbueva et al observed PAX5 expression in all the cases of this subtype included in his study. [44] In both the studies the blasts showed weak positivity when compared to the adjacent normal B cells. Zhang et al studied the expression of PAX5 various NHL and leukemia and concluded that though

PAX5 expression is noted in leukemia, their value in classifying these leukemia was minimal.[39]

Though the common understanding is that the PAX5 expression is lost in plasma cells differentiation, Pein Lim et al demonstrated PAX5 immunoexpression in a few cases of multiple myeloma. They were then suggested to be retained products of early stages of maturation. [45]

In addition to the lymphoid cells, neuroendocrine tumors also show positive PAX5 expression. [5][36] It is negative in case of carcinoid tumors but is positive in a high proportion of Merkel cell carcinomas and small cell carcinomas. The other non hematopoietic tumor that shows PAX5 expression is transitional cell carcinoma of urinary bladder.[46][47] The expression of PAX5 in these cases was found to be inversely proportional to the level of differentiation and directly proportional to the stage.[47] This increased expression of PAX5 in more aggressive forms suggests they have a prognostic value. PAX5 expression is noted in CNS tumors like medulloblastomas, astrocytomas, neuroblastomas etc. [48][49] Rare expression of PAX5 was noted by Tarlakovic et al and Paulette et al.[36][50]

The aberrant expression of PAX5 in tumors like neuroendocrine carcinomas, transitional cell carcinomas, some CNS tumors and T cell lymphomas indicate a possible role of PAX5 in tumorigenesis. Stewart et al proposed that the PAX5

suppresses the transcription of p53, a tumor suppressor gene and thereby leading to development of the tumor.[48]

The translocation t (9;14) involving the PAX5 region is found to be linked to lymphoplasmacytic lymphoma, further implying the role of PAX5 in tumorigenesis.[51] Balasenthil et al postulated that regulation of PAX5 by metastasis associated protein (MTA1) lead to its over expression and lymphoma genesis.[52]

Gilles A Roubischad et al identified about five isoforms of PAX5 from normal and lymphoma cells. They observed that all these isoforms had different sequence and transactivation properties. PAX5 FL isoform was noted predominantly in the lymphomas. Thus this altered form of PAX5 may be the reason behind the development of lymphoma in these cases. Moreover the oncogenic role of PAX5 in certain T cell lymphomas has been demonstrated in mice.[53]

The high expression of PAX5 in lymphomas, certain non haematolymphoid malignancies and its possible role in the development of these tumours have encouraged the investigators to develop a targeted therapy against PAX5. Mengyong et al have managed to generate a cytotoxic T lymphocyte lines against a sequence referred to as TLP on the PAX5 peptide. They observed a significant growth restriction and destruction of tumour cells expressing high PAX5 in mice. Though the in vivo testing has not been done in humans PAX5

poses to be an ideal target for immunotherapy in patients having tumors with aberrant expression of PAX5.[54]

In spite of its expression in various non haematolymphoid tissues and tumors PAX5 is still an excellent marker for B cell lineage. Paulette et al and Kirsten et al observed a highly specific immunoexpression of PAX5 in B cell lymphomas.[50][6] Neuroendocrine carcinomas were the only other tumours that which showed high reactivity with PAX5 next to lymphomas. Less than 1% of carcinomas showed immunoreactivity with PAX5.

MATERIALS AND METHODS

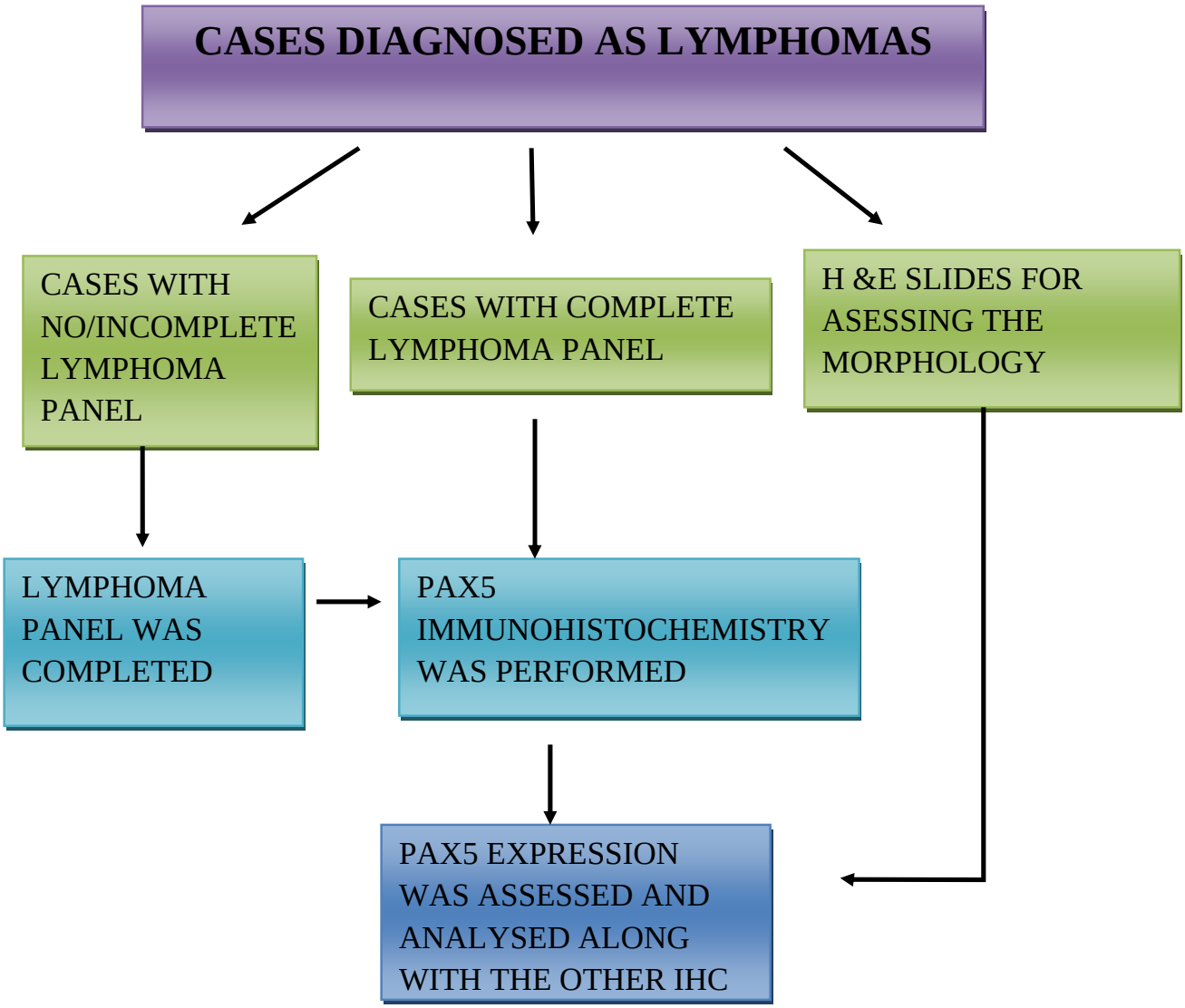
All the cases diagnosed as lymphomas from January 2009 to December 2010 in the department of pathology, PSG institute of medical science and research, Coimbatore were considered in the study. The cases with inadequate tissue for performing Immunohistochemistry were then excluded from the study.

The clinical data required for the study like the age, sex, site and stage of the tumor were retrieved from the medical records department of PSG institute of medical science and research after obtaining permission from the concerned authorities and institute human ethics committee clearance. The hemotoxylin and eosin slides of these cases were analysed for assessing the morphology and also typing.

The immunohistochemical slides which were used for classification of the lymphomas were also retrieved and analyzed. The common panel of markers included was CD45, CD3, CD20, CD15 and CD30. These were run for routine diagnostic reasons in the department. A few cases were the panel was not complete was completed during the study period. Paraffin blocks of those sections which had high tumor density were included for the study by reading the H and E slides. Blocks of slides which had less tumor material or extensive

necrosis were excluded. The following flowchart (figure 9) depicts the study plan .

Fig 9: THE STUDY PLAN



Immunohistochemistry was done using the supersensitive HRP detecting system. The following table (table 6) shows the clones of the various primary antibodies used during the study

TABLE 6: The markers, clone of the primary and controls used

MARKERS	COMPANY	CLONE	CONTROL
CD45	Biogenex	PD7/26/16	Lymphnode
CD3	Biogenex	PSI	Lymphnode
CD20	Biogenex	L-26	Lymphnode
CD15	Biogenex	BRA4F1	Kidney
CD30	Biogenex	HRS-4	Hodgkin's lymphoma
PAX5	Dako	DAK	Tonsil

The procedure for Immunohistochemistry was similar for all the markers and it is as follows.[19]

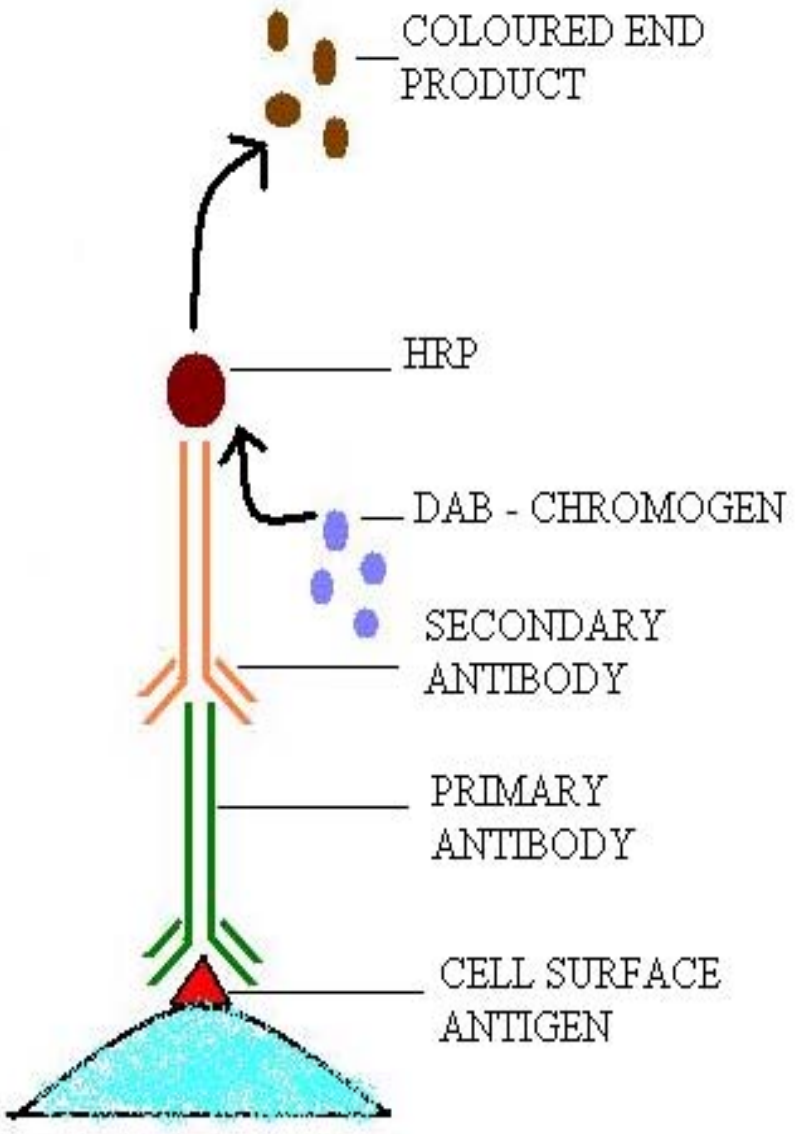
PRINCIPLE: In an immunohistochemical reaction , the specific antigen present in the cells and tissues was detected in a two stage process, (Figure 10) that includes:

1) The binding of the primary antibody to its specific epitope in the tissues.

2) Detection of this bound antibody using a dextran polymer bound secondary antibody in a calorimetric reaction that involves a chromogen.

In this method, primary antibody to the specific antigen is first added and is then followed by the addition of a dextran polymer linked to multiple conjugated secondary antibodies and horse raddish peroxidase enzyme. This multiple secondary antibodies bound to the primary antibody and the signal is amplified by the use of a suitable chromogen 3, 3'diaminobenzidine tetra hydrochloride (DAB).

FIGURE 10: PRINCIPLE OF IHC USING HRP KIT



STEP 1:

Antigen retrieval:

This is a process to unmask the epitopes of the specific antigens that are masked by cross linking action of formalin during routine processing. There are various methods for antigen retrieval. The methods include,

1) Pressure cooker method

2) Microwave method

3) Proteolytic digestion method

Of the above, the pressure cooker method is used for the present study. Here, the tissue is exposed to the additive effects of both heat and pressure thereby bringing out the full antigenicity. After dewaxing and dehydrating in graded alcohols, the slides were subjected to antigen retrieval in a pressure cooker for 10 minutes with EDTA buffer at pH 9.

Reagents used:

- EDTA buffer at pH 9
- 3% hydrogen peroxide (H2O2) in distilled water - To block endogenous peroxidase activity inorder to prevent nonspecific background staining

- 0.01M Phosphate buffered saline (PBS) with a pH value of 7.6. It was prepared by dissolving the following substances in 1000 ml of distilled water.

  1.Na2HPO4 Dibasic sodium phosphate, anhydrate 17.5g

  2.KH2PO4 Monobasic potassium phosphate, anhydrous 2.5g

  3.NaCl Sodium chloride 17.0g

- **Blocking reagent**- casein in PBS with 15mM sodium azide. This was used to blocks non specific protein binding.

- **Step 2**:

- **Primary antibodies**. All the primary antibodies used in the study were in ready to use formulation

- **Step 3**:

- **Poly HRP reagent**- anti-mouse and anti-rabbit IgG complex linked to Horse radish peroxidase enzyme.

- **Step 4**:

- DAB (3, 3'Diamino Benzidine tetra hydrochloride) - Chromogen.

  It offers great sensitivity as an HRP calorimetric chromogen and provides insoluble permanent coarse brown precipitate.

Step 5

- Harris hematoxylin as counter stain.
- DPX (Distrene dibutyl phthalate Xylene) - Mountant.

PROCEDURE:

Immunohistochemical Staining with the two specific antibodies were done as follows

- Slides were deparaffinised
- deparaffinised slides were hydrated using graded alcohol.
- Antigen retrieval: using EDTA buffer at pH 9.0 in a pressure cooker for 10 minutes.
- Fast cooling under tap water.
- Washed in PBS buffer at pH 7.6 for for 5 minutes
- After wiping off excess PBS buffer the slides were immersed in 0.3% H_2O_2 for 20 minutes to block endogenous peroxidase activity.
- Washed in PBS buffer thrice, each 5 minutes
- Slides were incubated in blocking solution for 10 minutes to block non-specific protein binding.

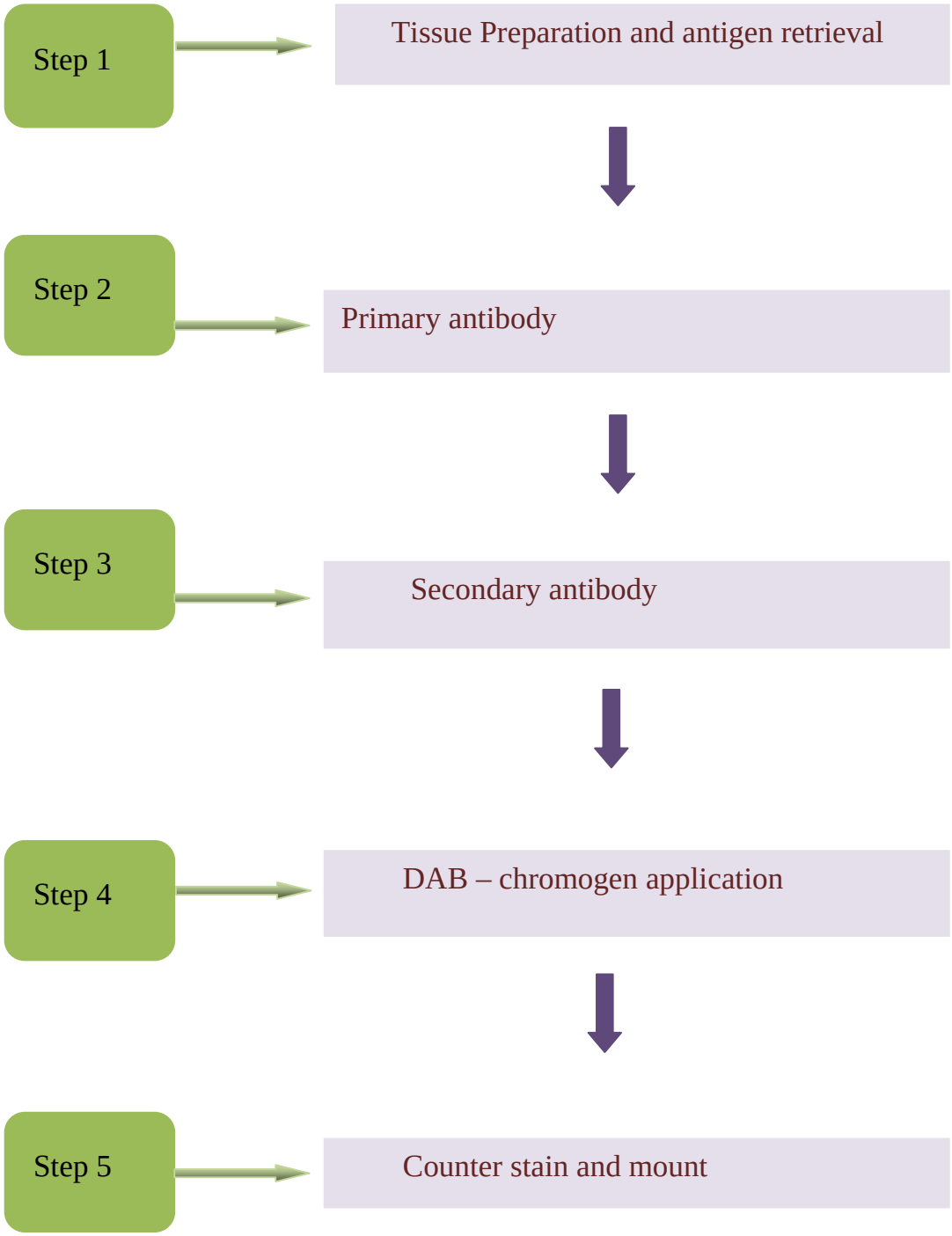
- Washed in PBS buffer thrice , each 5 minutes.
- Slides were incubated with the primary antibody for 1 Hr.
- To enhance the signal intensity, the sections were put in superenhancer for 30 minutes.
- Washed in PBS buffer thrice, each 5 minutes.
- Horse radish peroxidase polymer reagent was added to the slide and incubated for 30 minutes.
- Washed in PBS buffer thrice, each 5 minutes.
- Chromogen Diamino Benzidine (DAB) was applied for 8 minutes.
- Washed in PBS buffer thrice, each 5 minutes.
- Sections were **counter stained with Harris hematoxylin** for 1 minute.
- Washed in tap water.

Sections were cleared in Xylene and mounted with DPX mountant.

These sections were than assessed for the immunoreactivity. All the CD markers were considered positive when they showed a membranous positivity in the neoplastic cells. Slides stained with PAX5 immunohistochemistry were considered positive when they show a brisk nuclear positivity. Less than 10% of

cells showing positivity were considered negative. The intensity of the staining was also considered.

Figure 11：Algorithm of the steps involved in running Immunohistochemistry



RESULTS

The department of pathology, PSG institute of medical science and research has received 8207 biopsy specimens over a period of two years from 1st January 2009 to 31st December 2010. The total number of malignancies reported during the study period was 922. Out of these 922 cases, 59 cases were reported as malignant lymphomas giving an overall incidence of 6.3%. Table 7 shows the no of malignancies reported each year and the incidence of lymphomas for the year 2009 and 2010.

TABLE 7: Incidence of lymphomas during the study period

YEAR	NO: OF MALIGNANCIES	NO:OF LYMPHOMAS	INCIDENCE RATES
2009	443	30	6.7
2010	479	29	6.0
TOTAL	922	59	6.3

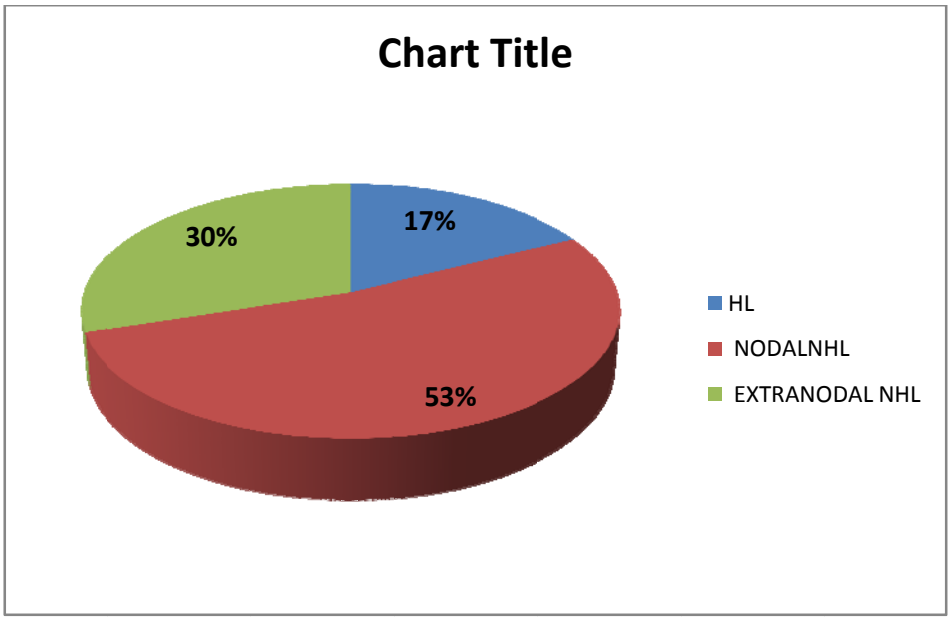
Of the 59 cases reported as lymphomas during the study period, 19 cases were not included in the study owing to inadequate tissue samples and unavailability of tissue blocks. The remaining 40 cases include 28 nodal lymphomas and 12 extra-nodal lymphomas of which 33 were non Hodgkin's lymphoma and 7 were Hodgkin's lymphoma. Most of the non Hodgkin's lymphomas were of B cell type. 4 out of the 33 non Hodgkin's lymphomas were not classified under a specific cell type and were

reported as unclassifiable. Table 8and Chart 1 show the various types of lymphomas included in the study and its % incidence distribution.

TABLE 8 – Distribution of lymphomas included in the study

TYPE	NO: OF CASES
HODGKINS LYMPHOMA	7
NONHOGKINS LYMPHOMA	
NODAL	21
EXTRA NODAL	12
TOTAL	**40**

CHART 1 – Distribution of lymphomas with % incidence



The age group ranged from 3 years to 75 years, with a mean age of 43.5 years. 5 out of 7 cases of Hodgkin's lymphoma were in the second and the fifth decades. 18 out of the 33 cases of non hodgkins lymphoma were in the sixth and seventh decade of life.

Table 9 shows the age wise distribution of various lymphomas.

Table 9- Distribution of various lymphomas age wise

AGE	**HL**	**NHL**		
		B CELL	**T CELL**	**UNCLASSIFIED**
0-10	1	0	1	1
11-20	3	1	1	0
21-30	0	0	2	0
31-40	0	0	0	1
41-50	2	5	1	0
51-60	0	6	1	0
61-70	1	8	2	1
>70	0	1	0	1
TOTAL	7	21	8	4

Of all the 40 cases, 28 were males and 12 were females, giving rise to a male: female ration of 2.3:1. All the cases of Hodgkin's lymphoma were males. B cell type non Hodgkins lymphoma were more common than the T cell type non Hodgkin lymphoma

in both the sexes. Chart 2, chart 3, table 10 and table 11 depicts the distribution of various lymphomas across different age groups and sex, which clearly shows a predilection for males.

Table 10 – Sex wise distribution of various lymphomas across various age groups

AGE	0-10	11-20	21-30	31-40	41-50	51-60	61-70	>70	TOTAL
MALE	2	4	1	0	4	7	9	1	28
FEMALES	1	1	1	1	4	0	3	1	12

CHART 2 - Sex wise distribution of lymphomas across various age groups

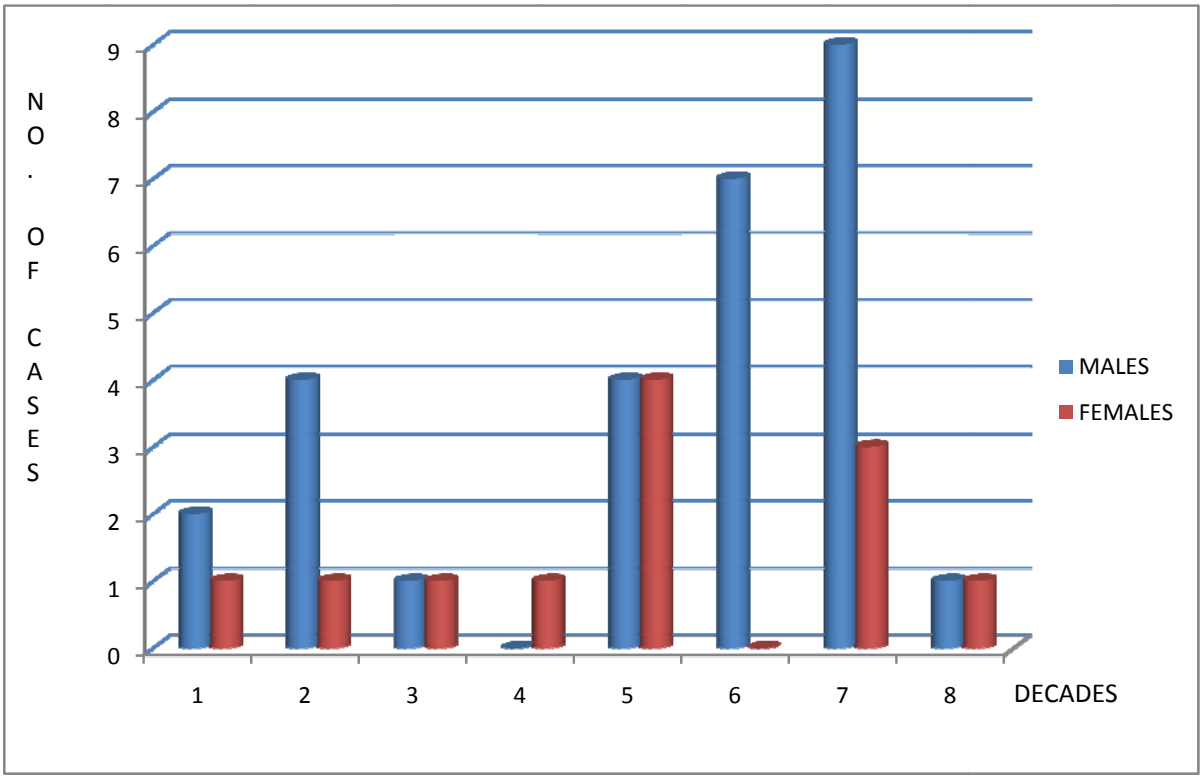
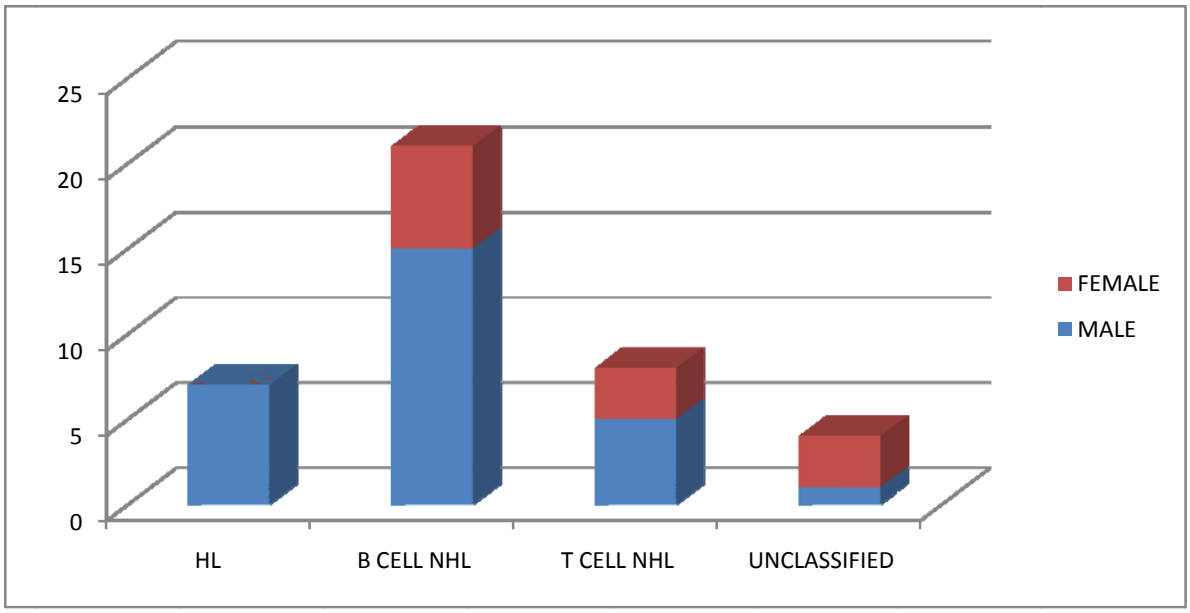


Table 11 – Distribution of lymphomas sex wise

SEX	HL	B CELL NHL	T CELL NHL	UNCLASSIFIED
MALE	7	15	5	1
FEMALE	0	6	3	3

Chart 3- Distribution of lymphomas sex wise

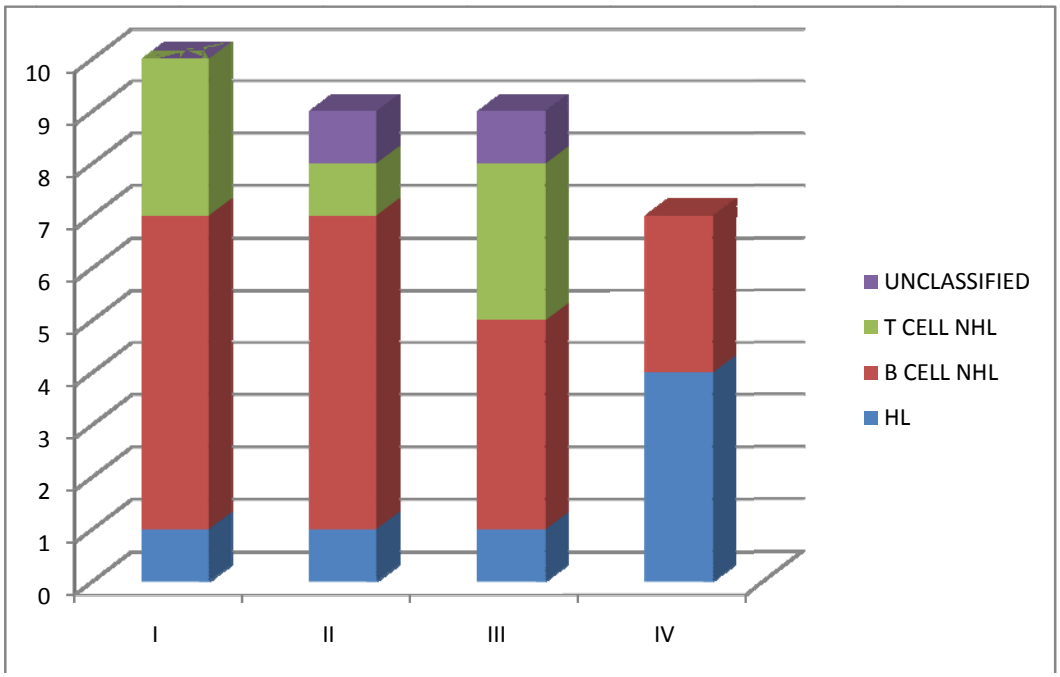


4 out of 40 cases could not be staged due to unavailability of necessary data. The lymphomas were staged based upon the Ann-Arbor staging into stage I, stage II, stage III and stage IV. Table 12 and chart 4 depicts the distribution of various lymphomas across different stages.

Table 12– Distribution of lymphomas across different stages

STAGE	HL	B CELL NHL	T CELL NHL	UNCLASSIFIED	TOTAL
STAGE I	1	6	3	0	10
STAGE II	1	6	1	1	9
STAGE III	1	4	3	2	10
STAGE IV	4	3	0	0	7

Chart 4 - Distribution of lymphomas across different stages



Sections of lymphomas were stained with the immunohistochemical markers, commonly used in the diagnosis of lymphomas which includes CD45, CD3, CD20,CD15,CD30. They were studied and determined whether positive or negative. Areas staining the reactive lymphoid cells were excluded.

All the cases except one were positive for CD45. This case had the following immunoprofile – CD45-ve, CD3-ve, CD20-ve, CD15-ve, CD30+ve and EMA+ve. Based on the morphology and the immunoprofile, it was diagnosed as anaplastic large cell lymphoma, a T/NK cell non Hodgkin's lymphoma.

The Reed-Sternberg cells of all the cases of Hodgkin's lymphoma were positive for CD15, CD30 (figure 13 to 15) and negative for CD3, CD20. This is the immunoprofile for classical Hodgkin's lymphoma.

Among the non Hodgkin's lymphoma, 7 were positive for CD3, 21 were positive for CD 20 and 4 were negative for both CD3 and CD20. Lymphomas positive for CD20 were classified as B cell non Hodgkin's lymphoma, those positive for CD3 were classified as T cell non Hodgkin's lymphoma and others were placed under unclassified category. Table 13 shows the number of cases in each immunoprofile.

Table 13 – IHC analysis of commonly used antibodies in the diagnosis of lymphomas

IMMUNOPROFILE	NO OF CASES
HL (CD 15 +VE, CD 30 +VE)	7
NHL(CD 15 –VE, CD 30 –VE)	
-CD 3 +VE	7
-CD20 +VE	21
-CD 45 +VE, CD 3 –VE, CD 20 –VE	4
-CD 45 –VE	1

All the cases were stained with PAX5 antibody and its expression in different age groups, sex, site, stage and types of lymphomas. A strong nuclear staining was considered positive.

23 out of the 40 cases were positive for PAX5. PAX5 was negative in all T cell non Hodgkin's lymphoma and the cases under the unclassified category. Out of 21 cases of B cell non Hodgkin's lymphoma, 18 were positive. Reed-Sternberg cells of Hodgkin's lymphoma were positive in 5 cases. (figure 16) Table 14 shows the expression of PAX5 in various lymphomas .

Table 14 – expression of PAX5in various lymphomas

TYPE OF LYMPHOMA	NO OF CASES	PAX 5 +VE	PAX 5 –VE
HL	7	5	2
NHL	33	18	15
-B CELL TYPE NHL	21	18	3
-T CELL TYPE NHL	8	0	8
-UNCLASSIFIED	4	0	4

PAX5 was positive in 17 out of 28 nodal lymphomas. This 17 cases consists of 5 cases of Hodgkins lymphoma and 12 cases of B cell type non Hodgkins lymphoma. Among the 12 extranodal lymphomas, 7 were of B cell type non Hodgkins lymphoma. PAX5 was positive in 6 of them. (figure 17 to20)Table 15 shows PAX5 expression in relation to site and type the of lymphoma.

Table 15 – Expression of lymphomas in relation to site and type of lymphoma

		TOTAL NO OF CASES	PAX 5 +VE CASES
NODAL LYMPHOMAS	HL	7	5
	B CELL NHL	14	12
	T CELL NHL	5	0
	UNCLASSIFIED	2	0
EXTRA NODAL LYMPHOMAS	HL	0	0
	B CELL NHL	7	6
	T CELL NHL	3	0
	UNCLASSIFIED	2	0

The expression of PAX5 in females and males were assessed. PAX5 was positive in 17 out of 28 male cases and 6 out of 12 female cases. All the B cell type non Hodgkins lymphoma of females were positive for PAX5. PAX5 was negative in 2 cases of Hodgkins lymphoma and 3 cases of B cell type non Hodgkins lymphoma of male.

Table 16 shows the expression of PAX5 in relation to sex

Table 16 – Expression of PAX5 in relation to sex

SEX	**HL**	**PAX 5+VE HL**	**B CELL TYPE NHL**	**PAX5 +VE B CELL TYPE NHL**
MALES	7	5	15	12
FEMALES	0	0	6	6

PAX5 expression in relation to age was studied and table 17 shows the observations in the expression of PAX5 in various age groups

Table 17- Expression of PAX5 in relation to age

AGE	HL	PAX 5 +VE HL	B CELL TYPE NHL	PAX 5 +VE B CELL TYPE NHL
0-10	1	1	0	0
11-20	3	1	1	0
21-30	0	0	0	0
31-40	0	0	0	0
41-50	2	2	5	5
51-60	0	0	6	4
61-70	1	1	8	8
>70	0	0	1	1

PAX5 expression across various stages was assessed. PAX5 was positive in most of the B cell non Hodgkin's lymphoma and Hodgkin's lymphoma regardless of the stage.

Table 18 – expression of PAX5 across various stages

STAGE	HL	B CELL TYPE NHL	PAX 5 +VE CASES
I	1	6	7
II	1	6	6
III	1	4	3
IV	4	3	6

DISCUSSION

Malignancies constituted 11.2 % (922/8207) among all biopsies reported from PSGIMSR during the period of study. Lymphomas constituted 6.3% of all malignancies in this institute which is significantly higher than that reported in the western literature (3%) and from the Chennai (4.25%) as reported in the national cancer registry atlas in the year 2001- 2003. [2]This increase in incidence observed in this institute could be due to the fact that this hospital is a tertiary care referral centre and is equipped with better diagnostic facilities. However an epidemiological survey and survey of lymphoma registry of the region is essential for proving the increased incidence noted in our study.

In B cell development, activation and differentiation PAX5 plays a crucial role especially when the neoplasm is both CD3 and CD20 negative. [38] Pax5 is expressed in all B cells except the terminal B cell population including the plasma cells.

Many studies quote that a significant number of cases which are CD20 negative express PAX5. A study done by Torlakovic et al states that PAX5 transcription factor expression studies play a vital role in the diagnosis. [38]

With the increase in the incidence of lymphomas globally an increased incidence observed in our institute prompted us to carry out this study on the significance of PAX5 expression in lymphomas.

We identified and retrieved 40 cases of lymphomas from the archives of pathology using the inclusion and exclusion criteria as discussed earlier. Most of the cases included in our study had the basic diagnostic panel of routine markers run for subtyping into B using CD20 and T cell type using CD3 / Hodgkin disease.

We had 7 cases of Hodgkin lymphoma and 33 cases of non Hodgkin lymphoma. Out of these 33 cases of non Hodgkin lymphoma 21 were from nodal and 12 cases were from extra nodal sites.

21/33 cases of NHL were of B cell type,8 were T cell type and 4 were unclassified.4 unclassified over 33 is a significant number and as the treatment and prognosis depends on the type of lymphomas an additional marker like PAX 5 is essential to identify the pre B cell types which are CD20 negative.

The peak age incidence of lymphoma observed in our study is 5^{th} to 7^{th} decade and is similar to all other studies. Though PAX 5 expression does not have a significant correlation with the age at presentation it is noteworthy to observe that the 2 cases of lymphomas (1 B cell and 1 unclassified) were negative. As

the numbers are small a larger prospective study on PAX5 in correlation with the age is essential.

In our study comprising 40 cases there was a slight male preponderance (28 males and 21 females) with a ratio of 1.3:1 which is very similar to the literature.

An equal distribution of lymphomas were seen across the stages I, II and, III except stage IV, where we had 7 out of 40 cases. PAX 5 was expressed in 23 out of forty cases and therefore Pax5 expression is not stage dependent. All the CD3 positive cases were PAX5 negative which was as expected.

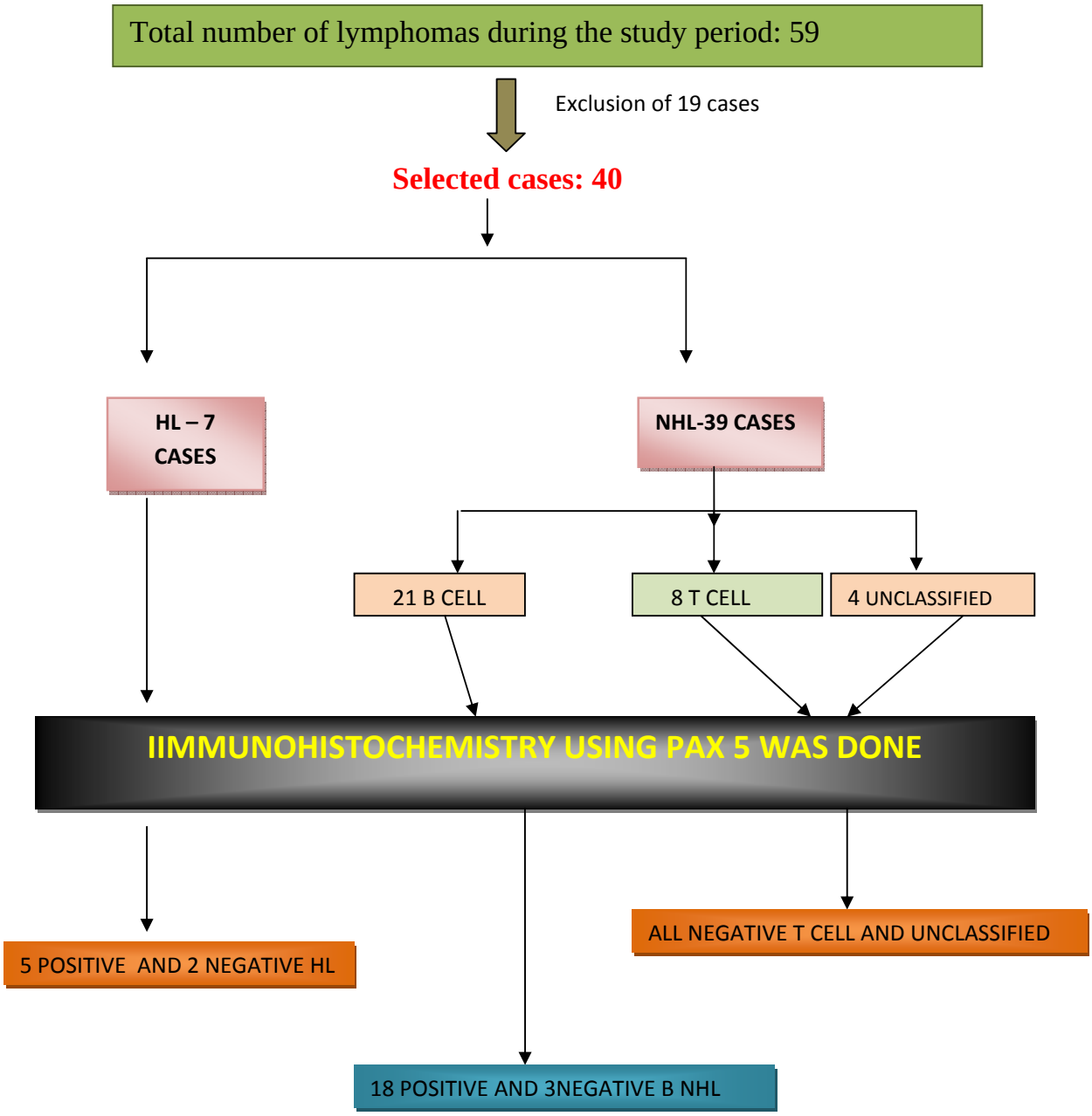
Out of the 21 CD20 positive cases only 18 cases where PAX5 positive and 3 cases were PAX5 negative. This negativity could be due to terminal B cell differentiation. We also observed in DLBCL a weak expression of PAX5 which is in correlation with the other studies. Out of the 7 cases of HL 5 expressed positivity with PAX5 . The literature states that HL usually shows a weak positivity with PAX5, but in our study 4 out of the 5 cases which expressed pax5 showed a strong intense reaction and in increased number of cells. As in most of the cases, the entire node is submitted for routine analysis; sampling error is highly unlikely in these cases. The strong reaction could be due to a neoplastic clone, which expresses increased PAX5 transcription factor

in our population. However the number of samples in our study is minimal, and hence a further larger study exclusively in HL is essential.

In conclusion , the analysis from our study showed there is a twofold increase in incidence of lymphoma in this institute when compared to the referred literature. NHL was more common than HL and the B cell phenotype was the commonest. Pax 5 had no significant correlation with age, sex or stage of the disease. 3 of the CD20 positive cases did not express PAX5, which could be due to a terminal B cell differentiation. We also found that in our study we showed that HL had a strong reaction to PAX5 when compared to other studies in the literature.

As our study showed a strong expression of PAX5 in HL, a larger study on PAX 5 expression in HL is vital to look for an increased expression in subset of Indian population.

Fig 12: Algorithm of the study



CONCLUSION AND SUMMARY

In conclusion our study on lymphomas reported from PSGIMSR from January 2009 to December 2010 revealed a 6.3% incidence which is 3% higher than that reported in the literature. Out of these 40 cases 17% were HL and 83% were NHL. We also infer that most of the B cell neoplasms expressed PAX5 as reported in the literature. Three cases of proven B cell neoplasms were PAX5 negative. This probably could be because of terminal differentiation in these cases. We also observed in our study, that 4 out of the 5 cases of HL which were positive for PAX5 showed a strong intense staining of nucleus with PAX5. A larger prospective study is essential to identify a subset of HL with increased PAX5 expression.

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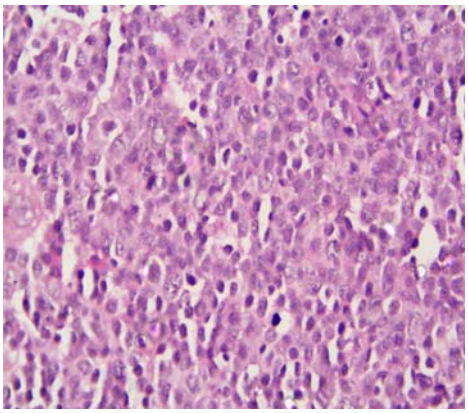

Fig17: Nodal B cell lymphoma (H&E, 40X)

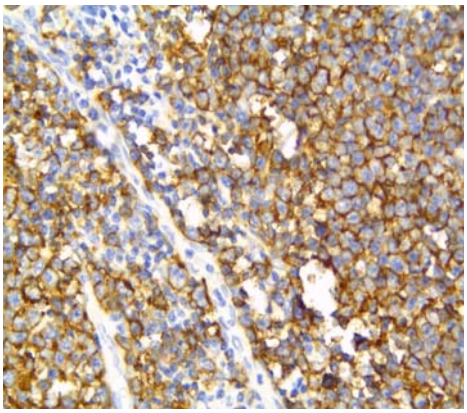

Fig18: A strong membranous positivity with CD20 (IHC,40X)

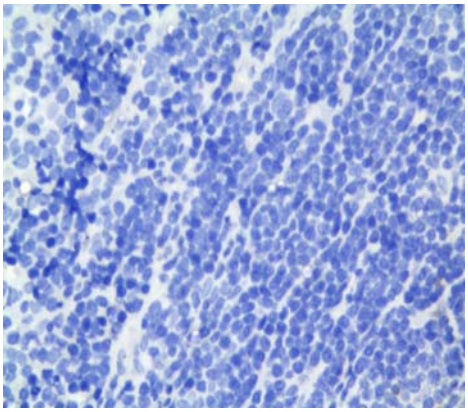

Fig19: Negative staining with CD3 NHL (IHC, 40X)

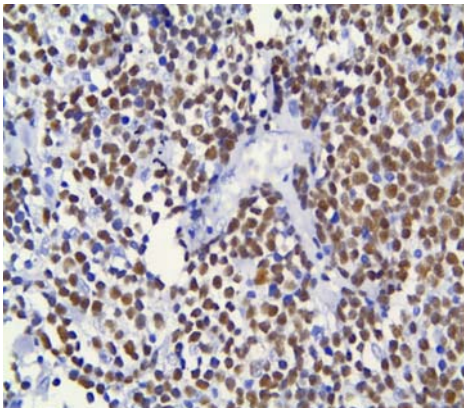

Fig20: A strong intense nuclear staining with PAX5 in a case of B cell NHL (IHC, 40X)

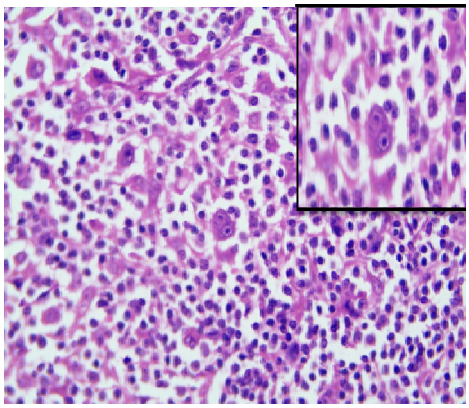

Fig13: Classical HL (H&E, 40X). Inset shows a classical RS cell

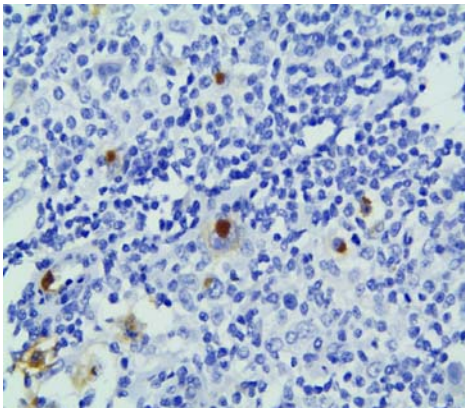

Fig14: RS cells showing a faint membranous positivity and a paranuclear dot like positivity with CD15 (IHC, 40X)

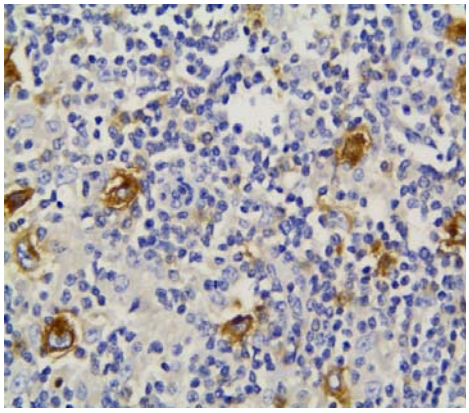

Fig15: RS cells showing a strong membranous positivity and a paranuclear dot like positivity with CD30 (IHC, 40X)

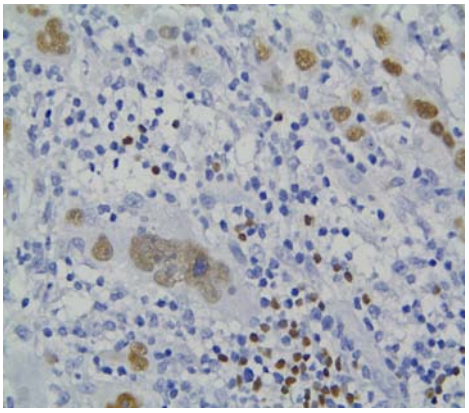

Fig16: RS cells showing a strong nuclear positivity with PAX5 (IHC, 40X)

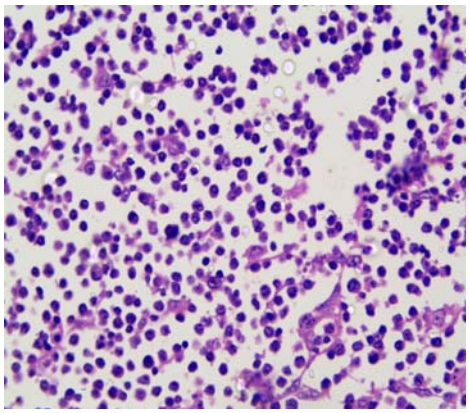

Fig21: T cell NHL (H&E, 40X)

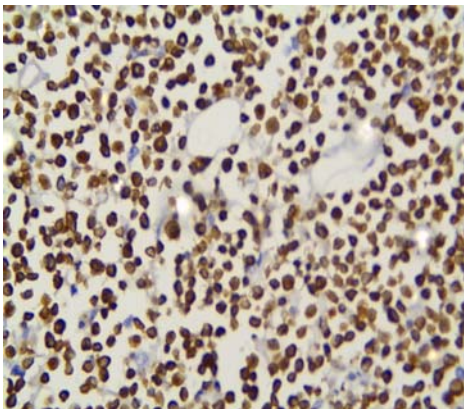

Fig22: A strong membranous positivity with CD3 in a case of T cell NHL (IHC, 40X)

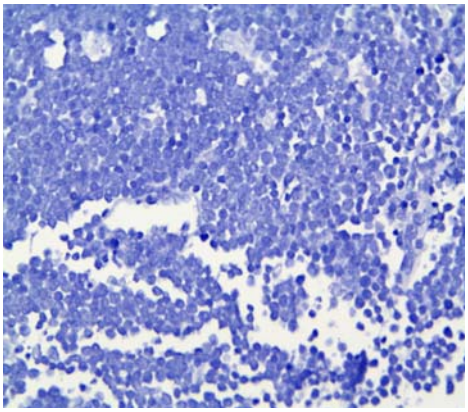

Fig23: Negative staining with CD20 in a case of T cell NHL (IHC, 40X)

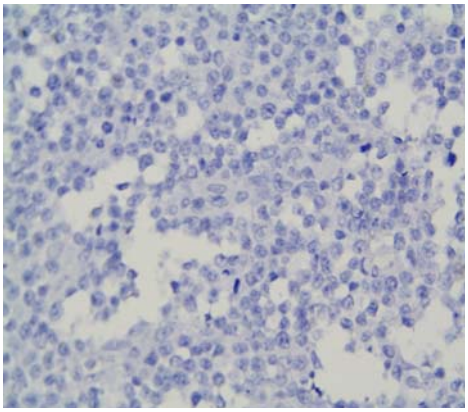

Fig24: Negative staining with PAX5 in a case of T cell NHL (IHC, 40X)

LIST OF ABBREVATIONS

ALCL –Anaplastic large cell lymphoma

BSAP – B cell specific activator protein

CD - Cluster of differentiation

CHL - Classical Hodgkin's lymphoma

DLBCL – Diffuse large B cell lymphoma

HL – Hodgkin's lymphoma

HD – Hodgkin's disease

IHC – Immunohistochemistry

LCA – Leucocyte common antigen

NHL – Non Hodgkin's lymphoma

NLPHL – Nodular lymphocyte predominant Hodgkin's lymphoma

PAX5 - Paired box 5

RS cells – Reed Sternberg cells

WHO – World health organisation

S.NO	BIOPSY NO.	AGE	SEX	CD 45	CD 3	CD 20	CD 15	CD 30	PAX 5	REPORT	SITE	STAGE
1	451/09	9	F	+	+	-	-	-	-	T-LYMPHOBLASTIC LYMPHOMA	AXILLARY LYMPH NODE	III
2	673/09	61	F	+	-	+	-	-	+	NHL	LYMPH NODE	I
3	1048/09	61	M	+	-	+	-	-	+	NHL	LYMPH NODE IN PAROTID	II
4	1332/09	48	M	+	-	-	+	+	+	HL	CERVICAL LYMPHNODE	IV
5	1896/09	75	F	+	-	-	-	-	-	NHL	CERVICAL LYMPNODE	III
6	2444/09	60	M	+	+	-	-	-	-	ANGIOIMMUNOBLASTIC T CELL LYMPHOMA	LEFT INGUINAL LYMPH NODE	III
7	2445/09	56	F	+	-	+	-	-	+	SPLENIC MARGINAL ZONE B CELL LYMPHOMA	SPLEEN	I
8	3482/09	55	M	+	-	+	-	-	-	B CELL LYMPHOMA	SKIN	NA
9	3907/09	44	F	+	-	+	-	-	+	NHL	FALLOPIAN TUBE	II
10	4030/09	53	M	+	-	+	-	-	-	DIFFUSE B CELL NHL	ABDOMINAL MASS	III
11	4061/09	42	F	+	-	+	-	-	+	NHL	OROPHARYNX	NA
12	4275/09	55	M	+	-	+	-	-	+	NHL, MANTLE CELL LYMPHOMA	TONSILS	II
13	4281/09	62	M	+	-	+	-	-	+	DIFFUSE LARGE B CELL LYMPHOMA	SUPRACLAVICULAR LYMPH NODE	II
14	4424/09	3	F	+	-	-	-	-	-	LYMPHOMA	CERVICAL LYMPH NODE	II
15	4647/09	63	M	+	-	+	-	-	+	LYMPNOMA	RETROPERITONEAL MASS	I
16	4892/09	48	F	+	-	+	-	-	+	DIFFUSE LARGE B CELL LYMPHOMA	AXILLARY LYMPH NODE	IV
17	154/10	4	M	+	-	-	-	-	-	SMALL ROUND CELL TUMOUR	MAXILLARY MASS	III

18	519/10	18	M	+	-	+	-	-	-	LYMPNHOMA	MEDIASTINAL MASS	IV
19	585/10	66	M	+	-	+	-	-	+	MALTOMA	GASTRIC BIOPSY	I
20	852/10	47	F	+	-	+	-	-	+	DIFFUSE LARGE B CELL LYMPHOMA	CAECUM,BIOPSY	II
21	902/10	75	M	+	-	+	-	-	+	SMALL LYMPHOBLASTIC LYMPHOMA	AXILLARY LYMPH NODE	III
22	971/10	50	M	+	+	-	-	-	-	T CELL LYMPHOMA	CERVICAL LYMPH NODE	I
23	1254/10	43	M	+	-	+	-	-	+	FOLLICULAR LYMPHOMA	POSTERIOR TRIANGLE LYMPH NODE	IV
24	1489/10	19	M	+	-	-	+	+	+	HODGKINS LYMPHOMA	CERVICAL LYMPH NODE	IV
25	1567/10	65	M	+	-	+	-	-	+	MANTLE CELL LYMPHOMA	CERVICAL LYMPH NODE	I
26	1690/10	16	M	+	-	-	+	+	-	HODGKINS LYMPHOMA	SUPRACLAVICULAR LYMPH NODE	III
27	1760/10	62	F	+	-	-	-	-	-	NHL	SKIN	NA
28	1809/10	15	M	+	-	-	-/+	+	-	HODGKINS LYMPHOMA	CERVICAL LYMPH NODE	II
29	1902/10	65	M	+	+	-	-	-	-	ATYPICAL LYMPHOID CELLS	SPLEEN	II
30	2011/10	65	M	+	+	-	-	-	-	MYCOSIS FUNGOIDES	SKIN	NA
31	2336/10	51	M	+	-	+	-	-	+	MANTLE CELL LYMPHOMA	CERVICAL LYMPH NODE	III
32	2356/10	21	F	+	+	-	-	-	-	MYCOSIS FUNGOIDES	SKIN	NA
33	2849/10	6	M	+	-	-	+	+	+	HODGKINS LYMPHOMA	CERVICAL LYMPH NODE	IV
34	3179/10	19	F	-	-	-	-	+	-	ANAPLASTIC MARGINAL ZONE LYMPHOMA	SUPRACLAVICULAR LYMPH NODE	I

35	3363/10	65	F	+	-	+	-	-	+	DIFFUSE LARGE B CELL LYMPHOMA	CERVICAL LYMPH NODE	I
36	3375/10	65	M	+	-	-	-	+	+	HODGKINS LYMPHOMA	AXILLARY LYMPH NODE	I
37	3472/10	62	M	+	-	+	-	-	+	B CELL NHL	CERVICAL LYMPH NODE	II
38	3667/10	21	M	+	+	-	-	+	-	ANAPLASTIC LARGE CELL LYMPHOMA	AXILLARY LYMPH NODE	III
39	4074/10	59	M	+	-	+	-	-	+	B CELL NHL	AXILLARY LYMPH NODE	III
40	4279/10	45	M	+	-	-	-	+	+	HODGKINS LYMPHOMA	AXILLARY LYMPH NODE	IV